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Review

Hormonal and Allosteric Regulation of the Luteinizing Hormone/Chorionic Gonadotropin Receptor

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Abstract

Luteinizing hormone (LH) and human chorionic gonadotropin (CG), like follicle-stimulating hormone, are the most important regulators of the reproductive system. They exert their effect on the cell through the LH/CG receptor (LHCGR), which belongs to the family of G protein-coupled receptors. Binding to gonadotropin induces the interaction of LHCGR with various types of heterotrimeric G proteins (G_s, $G_{q/11}$, G_i) and β-arrestins, which leads to stimulation (G_s) or inhibition (G_i) of cyclic adenosine monophosphate-dependent cascades, activation of the phospholipase pathway ($G_{q/11}$), and also to the formation of signalosomes that mediate the stimulation of mitogen-activated protein kinases (β-arrestins). The efficiency and selectivity of activation of intracellular cascades by different gonadotropins varies, which is due to differences in their interaction with the ligand-binding site of LHCGR. Gonadotropin signaling largely depends on the status of *N*- and *O*-glycosylation of LH and CG, on the formation of homo- and heterodimeric receptor complexes, on the cell-specific microenvironment of LHCGR and the presence of autoantibodies to it, and allosteric mechanisms are important in the implementation of these influences, which is due to the multiplicity of allosteric sites in different loci of the LHCGR. The development of low-molecular-weight allosteric regulators of LHCGR with different profiles of pharmacological activity, which can be used in medicine for the correction of reproductive disorders and in assisted reproductive technologies, is promising. These and other issues regarding the hormonal and allosteric regulation of LHCGR are summarized and discussed in this review.

Keywords: luteinizing hormone; human chorionic gonadotropin; luteinizing hormone/chorionic gonadotropin receptor; allosteric regulator; allosteric site; adenylyl cyclase; β-arrestin; biased signaling; steroidogenesis; ovulation

1. Introduction

The most important components of the signaling system involved in the regulation of the functional activity of the male and female reproductive system are gonadotropins, such as luteinizing hormone (LH), chorionic gonadotropin (CG) and follicle-stimulating hormone (FSH), and their receptors. LH and CG, produced by gonadotrophs of the adenohypophysis (LH, and sulphated CG), as well as the embryo and placenta during the first trimester of pregnancy (hyperglycosylated and classical CGs), are endogenous ligands of one G protein-coupled receptor (GPCR), which is accordingly called the LH/CG receptor (LHCGR). They bind to a high-affinity orthosteric site located in the ectodomain of this receptor. This is not a common case for GPCRs, since most receptors in this superfamily are activated by a single orthosteric agonist, but for a number of GPCRs, most notably polypeptide hormone receptors, there are two or more orthosteric site ligands, as has been demonstrated for the melanocortin receptors [1], orexin receptors [2], apelin receptor [3], and *N*-formyl-peptide receptor [4]. In this case, the multiplicity of ligands can be due to various variants of proteolysis or modification of the prohormonal molecule, a striking example of which is the site-specific proteolysis of pro-opiomelanocortin, as well as cross-interaction, which well illustrates the interaction of different orexin isoforms with their receptors. The multiplicity of ligands is even more characteristic of receptor tyrosine kinases, which is most clearly demonstrated by the receptors of the insulin family peptides [5], the receptors of the epidermal growth factor receptor family [6] and different isoforms of vascular endothelial growth factor (VEGF) receptor [7].

LH and human CG (hCG) bind to LHCGR with high affinity, causing a wide range of physiological responses, which is due to the functional interaction of activated LHCGR with a large number of transducer proteins, through which the regulation of many intracellular targets is carried out. Despite the fact that the regulatory effects of LH and hCG are realized through the same receptor, and the high-affinity orthosteric site responsible for their binding has a similar structure and topology, the specificity of activation of intracellular cascades by gonadotropins, and, accordingly, the cellular response caused by them can vary significantly [8–17]. This has a very definite biological significance, taking into account the different physiological roles of LH and hCG in humans and some other mammals. No less interesting is the fact that each of these hormones has a large number of isoforms that differ in their specific activity, and this is largely determined by the characteristics of the glycosylation of their molecules, which significantly

affects the binding of gonadotropin to LHCGR and the bias of signal transduction [18–23]. Finally, the response to gonadotropin may be regulated at the level of LHCGR through post-translational modifications of the receptor, as well as through receptor complex formation, including heterodi(oligo)merization with other receptors, most notably the FSH receptor (FSHR) [14,24–30]. Glycosylation of gonadotropins and the processes of complex formation and modification of LHCGR are the most important factors in the control of LH/CG-mediated signal transduction in target cells, and they are based on allosteric mechanisms that determine both the affinity of the gonadotropins for LHCGR and the stability and pattern of activated conformations of LHCGR responsible for selective signal transduction to intracellular effectors.

However, other, less studied factors in this aspect, such as the physiological state of the organism, as well as the pathological processes, including lipotoxicity, endoplasmic reticulum stress and overproduction of reactive oxygen and nitrogen species, also have a significant impact on LH/CG-mediated signal transduction, disrupting post-translational modification and "maturation" of LHCGR and other signal proteins. In animal models of type 1 and type 2 diabetes mellitus with hyperglycemia, insulin signaling dysfunctions, redox imbalance and increased inflammatory processes, we and other authors have shown a significant decrease in both *LHCGR* gene expression and the number of functionally active LHCGRs on the surface of testicular and ovarian cells, which led to weakened LH/CG signaling and impaired steroidogenesis [31–35].

All these factors, to one degree or another, influence the lipid composition of membranes, the ionic and amino acid composition in the intra- and intercellular environment, and the availability and activity of adapter and regulatory proteins capable of forming complexes with LHCGR. It is known that cholesterol and phospholipids, some simple ions (Na^+, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cl^-, and others), amino acids and their derivatives (Tyr, Phy, Trp, Leu, Ile, homocysteine, agmatine and others) can function as allosteric modulators of GPCRs [36–42]. Their regulatory, modulating effect on the activity of LHCGR and other components of LH/CG-stimulated cascades cannot be excluded, although strong evidence for this has not yet been obtained. Autoantibodies to gonadotropins and LHCGR, the formation of which has now been proven, can act as endogenous allosteric regulators [43–47]. They are able to function as allosteric regulators of LHCGR, which have their intrinsic activity, and to modulate the effects of gonadotropins. It should be noted that with regard to other GPCRs, there are numerous works on the allosteric effects of autoantibodies to GPCRs and their key role in the development of autoimmune diseases [48–51].

Thus, there are many mechanisms and targets of allosteric regulation of LHCGR and the signaling pathways realized through it, which indicates the possibility of fine-tuning the intensity and selectivity of LH/CG-induced signal transduction. This tuning depends on the physiological status of the target cell, the pattern of LH and CG glycoforms, the composition and ratio of LHCGR-containing complexes, the activity of other signaling cascades modulating LHCGR activity, and the presence of autoantibodies to gonadotropins and LHCGR. Such a variety of allosteric influences is predetermined by the existence of a large number of allosteric sites in the LHCGR, as has been shown for other class A GPCRs. These sites can be localized in various loci of the receptor molecule, including in the extracellular loops (ECLs) and the external entrance to the transmembrane tunnel, in the internal cavity of this tunnel, on the outer surface of the transmembrane domain (TMD), which is in contact with the lipid bilayer of the membrane, and in the intracellular loops (ICLs) and in the cytoplasmic entrance to the transmembrane tunnel [42] (Fig. 1). Accordingly, it becomes possible to develop site-directed allosteric ligands that will have a different profile of pharmacological activity, being both modulators of the effects of gonadotropin and having their intrinsic agonistic or antagonistic activity. As is known, allosteric regulators can reduce (negative allosteric modulator, NAM) or increase (positive allosteric modulator, PAM) the affinity and/or effectiveness of an orthosteric agonist, do not directly affect these parameters, but modulate other allosteric effects (silent allosteric modulator, SAM), exhibit its intrinsic activity as a full agonist, inverse agonist or neutral antagonist in the absence of an orthosteric agonist, as well as combine the activity of a full agonist or antagonist with the activity of PAM (ago-PAM, PAM-antagonist) or NAM (ago-NAM) [42,52–56] (Table 1). Since allosteric sites functionally interact not only with the orthosteric site, but also with each other, forming a multidirectional network of such interactions, the activity profile of the allosteric ligand can be very complex and cannot be described within the framework of the proposed classification, which is to a certain extent true for some low-molecular-weight (LMW) allosteric regulators of LHCGR.

In addition, given the multiplicity of signaling cascades activated by orthosteric agonists through GPCRs, allosteric modulators can selectively enhance or, conversely, attenuate only a certain signaling cascade, thereby demonstrating biased allosteric modulator (BAM) activity [54,56,57]. Such modulators are important for ensuring selectivity of action and achieving target physiological effects when using an orthosteric agonist that has low selectivity for intracellular signaling. Allosteric regulators with their intrinsic activity, full or inverse agonists, can also specifically regulate only a certain signaling cascade, functioning as biased allosteric full or inverse agonists (Table 1).

This review is devoted to the analysis and discussion of the similarities and differences in the regulatory effects of LH and various forms of hCG on the activity of LHCGR and its signaling pathways, the role of glycosylation of LH

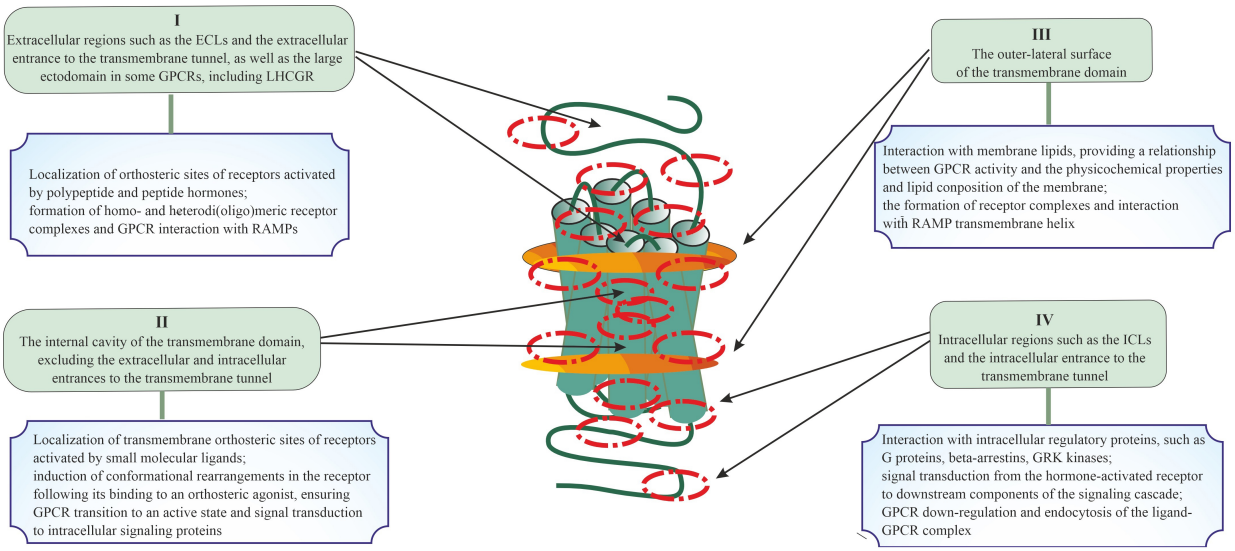


Fig. 1. Topologically distinct regions and domains of G protein-coupled receptors (GPCRs) in which allosteric sites can be located, and the role of these regions and domains in the signal transduction and the formation of di- and oligomeric complexes. Four possible localizations for allosteric sites of GPCRs are presented, including extracellular regions and/or domains (I), the transmembrane tunnel of the TMD (II) and its side surfaces that contact plasma membrane lipids (III), and cytoplasmic regions and/or domains (IV). The main functions of these topologically different regions or domains of GPCR in signal transduction and in the formation of various complexes (between GPCR protomers or between the GPCR protomers and other signal and adapter proteins) are presented, which indicates the possible role of allosteric sites located in them in the implementation of these processes. At the same time, taking into account the cross-talk between allosteric and orthosteric sites, as well as between allosteric sites, this role can vary greatly and be much broader and more diverse. ECLs, extracellular loops; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; RAMPs, receptor activity-modifying proteins; ICLs, intracellular loops; GRK, G protein-coupled receptor kinase; TMD, transmembrane domain.

and CG and LHCGR complex formation in signal transduction, including its heterodimerization with FSHR, as well as the possible contribution of autoantibodies against gonadotropins and LHCGR in the control of LHCGR activity and in the etiology and pathogenesis of reproductive dysfunctions. Along with this, the review examines modern advances in the development of allosteric regulators of LHCGR, including full and inverse LMW agonists and allosteric modulators capable of interacting with the transmembrane allosteric site of LHCG. The regulators with agonistic activity are of significant interest for the correction of hypogonadotropic conditions and in the assisted reproductive technologies (ARTs), while regulators with antagonistic activity may be in demand in the treatment of hormone-dependent tumors and in contraception. The discussion of the issues presented above is preceded by a brief description of the structural and functional organization of gonadotropins and their receptors, the molecular basis of their interaction, as well as the LHCGR signaling realized through G proteins and β-arrestins.

2. Structure of Luteinizing Hormone and Chorionic Gonadotropins

The LH is a $\alpha\beta$-heterodimer with a molecular weight of about 30 kDa. LH secretion is carried out by gonadotrophs, specialized cells of the adenohypophysis, and is controlled by the hypothalamic gonadotropin-releasing hormone (GnRH) [58–60]. Along with GnRH, the synthesis and secretion of LH are regulated by polypeptide factors such as kisspeptin, melanocortin peptides, gonadotropin-inhibiting hormone, leptin, adiponectin, activins and inhibins, as well as steroid hormones, corticosteroids and, by a negative feedback mechanism, sex steroid hormones, primarily estrogens, and some growth factors and cytokines [61–69]. These factors can act either at the level of control of GnRH release, directly or indirectly affecting the activity of GnRH-expressing neurons, or directly influence the synthesis and secretion of LH by pituitary gonadotrophs. Along with this, some of these regulators can influence both the secretion of GnRH and the production of LH, as shown, for example, for leptin [70,71] and gonadotropin-inhibiting hormone [72].

Among the isoforms of human CG (hCG), the isoform of hCG expressed in the pituitary gland, the so-called sulphated hCG [73], and the classical hCG, which has an extrapituitary origin, are known. Classical hCG is synthesized and secreted by the embryo and placenta during the first trimester of pregnancy. Sulphated hCG is produced by the gonadotrophs of both men and non-pregnant women, and despite its small quantities, it is significantly more active than LH, and thus makes a significant contribution to the

Table 1. Pharmacological profile of ligands of G protein-coupled receptors (GPCRs) allosteric sites, including allosteric modulators of the effects of orthosteric agonist, allosteric regulators with their intrinsic activity and with a combination of their intrinsic and modulating activity.

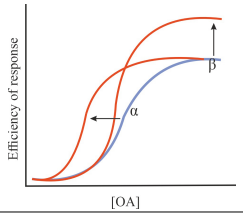
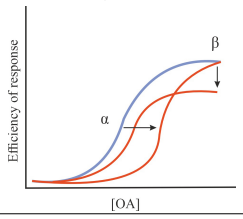
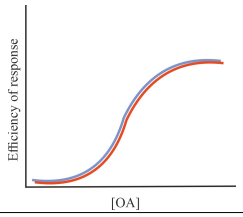
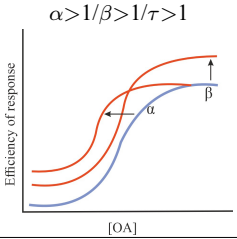
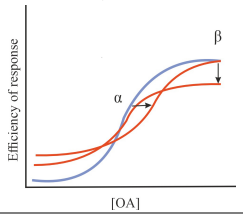
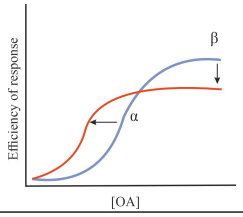
Ligand type	Effect on the basal and stimulated GPCR activity	Characteristics of GPCR binding and/or activation
Positive, negative, silent and biased allosteric modulators (PAM, NAM, SAM, and BAM)		
PAM	Increase in the affinity of an orthosteric agonist (OA) for GPCR and/or in the effectiveness of its action without affecting the constitutive activity of GPCR	$\alpha>1/\beta>1/\tau=1$ 
NAM	A decrease in the affinity of OA for GPCR and/or in the efficiency of its action without affecting the constitutive activity of GPCR	$\alpha<1/\beta<1/\tau=1$ 
SAM	No effect on the affinity of OA for GPCR and (or) on the effectiveness of its action, possible change in some characteristics of the regulatory effect of allosteric agonists on GPCR activity (biased agonism, specificity of interaction with certain types of G proteins and β-arrestins, formation of receptor complexes, availability of GPCR for allosteric regulators), no effect on the constitutive activity of GPCR	$\alpha=1/\beta=1/\tau=1$ 
BAM	A change in the affinity of an OA for a GPCR and/or its efficiency of action, resulting in selective activation (positive allosteric modulator, PAM), inhibition (negative allosteric modulator, NAM) or modification (silent allosteric modulator, SAM) of a certain signaling cascade or a certain pattern of signaling cascades, which provides bias in OA action	The values of α, β and τ vary depending on the specific cascade and the nature of the modulating allosteric action
Allosteric regulators with intrinsic activity, such as full (Full Ago) or inverse allosteric agonists (Inv Ago) and neutral allosteric antagonists (Ant)		
Full Ago	Stimulation of GPCRs in the absence of OA or other allosteric agonist, no effect on OA's affinity for GPCRs or its effectiveness	$\alpha=1/\beta=1/\tau>1$ Characterized by preferential activation of a certain signaling cascade (biased agonism), and more moderate stimulation of GPCR compared to OA
Inv Ago	Reduction in constitutive GPCR activity in the absence of OA, as well as inhibition of OA- or allosteric agonist-stimulated GPCR activities	$\alpha=1/\beta=1/\tau<1$ Characterized by preferential suppression of a specific OA-stimulated signaling cascade, as well as a selective influence on the pattern of active states of constitutively active GPCR; more moderate inhibition of constitutive and OA-stimulated GPCR activity as compared to orthosteric site inverse agonists
Ant	Inhibition of OA- or allosteric agonist-stimulated GPCR activities without affecting constitutive GPCR activity	$\alpha=1/\beta=1/\tau<1$ Characterized by a moderately pronounced inhibitory effect on OA-stimulated signaling cascades, which does not lead to their blockade, typical of orthosteric site antagonists

Table 1. Continued.

Ligand type	Effect on the basal and stimulated GPCR activity	Characteristics of GPCR binding and/or activation
Allosteric regulators with combined modulating and agonistic activity (Ago-PAM – full allosteric agonist-PAM; Ago-NAM – full allosteric agonist-NAM; Ant/PAM – neutral allosteric antagonist/PAM)		
Ago-PAM	Increase in the affinity of OA for GPCR and/or in the efficiency of its action, stimulation of basal GPCR activity in the absence of OA, possibly potentiation of the effect of OA on GPCR activity	$\alpha>1/\beta>1/\tau>1$ 
Ago-NAM	Reduction in the affinity of OA for GPCRs and/or in the efficiency of its action, stimulation of basal GPCR activity in the absence of OA	$\alpha<1/\beta<1/\tau>1$ 
Ant/PAM	A decrease in the effectiveness of OA on GPCRs (antagonistic effect), but an increase in the affinity of OA for GPCRs (PAM effect)	$\alpha>1/\beta<1/\tau=1$ 

Note: α – the factor of binding cooperativity between the OA and allosteric modulator; β – the operational factor of cooperativity for quantitative evaluation of the effects of allosteric modulator on operational efficacy of OA (receptor activation); τ – operational efficacy for the complex of GPCR with allosteric ligand. The values of binding cooperativity (α) and operational cooperativity (β) greater than 1 denote positive cooperativity, and the corresponding values below 1 denote negative cooperativity. The value 1 for operational efficacy (τ) is normalized since the absolute value of τ can vary widely depending on the basic design parameters used. For BAM and other cases of biased allosteric regulation, different variants of the values of α, β and τ are possible, since in this case it is necessary to differentiate the factors being assessed for each specific signaling cascade regulated by OA or allosteric regulator. PAM, positive allosteric modulator; NAM, negative allosteric modulator; SAM, silent allosteric modulator.

total LH-like activity of the common pool of gonadotropins [73]. A hyperglycosylated form of the hormone has also been discovered, which is expressed at the early stages of embryo development, mainly at the cytotrophoblast stage and significantly differs in functional properties from LH and other forms of hCG [74,75].

The α-subunit is encoded by a single gene and is common to all gonadotropins. It is a polypeptide with a length of 116–120 amino acid residues (AARs) and is characterized by a high degree of homology of the primary structure. Thus, when comparing the human α-subunit with those of monkeys, up to 98% identity of the amino acid sequence was shown, when comparing with the α-subunits of rat, mouse, bovine, pig, dog, cat and rabbit, 75–76% identity was found, and when comparing with orthologues of birds, amphibians, reptiles, and fish only 70–73% identity was shown. β-Subunits vary greatly in primary structure and determine the type of gonadotropin. When comparing the β-subunits of human LH, hCG and FSH, only 33% of identical AARs were identified. At the same time, cysteine residues, which determine the 3D structure of β-subunits and are responsible for the formation of functionally active $\alpha\beta$-heterodimer complexes, are highly conserved. The degree of identity of the sequences 45–153 β-LH and 29–139 human β-hCG is significantly higher and amounts to 83%, which indicates their structural and functional similarity and determines the ability of these hormones to specifically bind to the same receptor. The homology of the β-subunits of LH or hCG varies among different animal species, but on average is quite high [18,76]. Thus, when comparing human and monkey β-LH, the identity of the primary structure varies only from 95 to 99%, and when comparing human β-LH with rat, mouse, rabbit and cat β-LH it decreases to 72–75%. Moreover, among various species of primates, the homology of β-LH and β-CG is noticeably higher than when compared with β-LH of other animals, which indicates a relatively late divergence of β-LH and β-CG in the evolution of higher vertebrates.

In the 1990s, based on X-ray diffraction data, the 3D structure of the heterodimeric hCG was first identified [77,78], and subsequently the 3D structure of the LH was established [79,80]. The main structural characteristic of the α- and β-subunits that make up gonadotropins is the presence of intramolecular disulfide bonds in them. They connect segments of α- and β-subunits that are distant from each other, causing them to cross each other and form a rigid knot structure called a cystine knot. Cystine knots are localized in the central part of the α- and β-subunits and stabilize three loops (L1, L2, and L3) extending from the center of the dimer. Two of them (L1 and L3) are rigid in structure and have the shape of a hairpin (the so-called hairpin structures), while the L2 loop is more flexible and is located on the opposite side from the center of the molecule [76,79]. In a heterodimer, the α- and β-subunits are located symmetrically with respect to each other, have an elongated shape and are characterized by a large ratio of surface area to volume of the molecule. The polypeptide, which corresponds to the *C*-terminal region of the β-subunit and extends beyond its central part, held together by cystine knots, functions as a safety belt by wrapping around the antiparallel α-helices that form the L2 loop of the α-subunit. The 3D structure of the seat belt is stabilized by an intramolecular disulfide bond, the formation of which involves cysteine residues, one of which is localized in the L1-loop of the β-subunit, and the other is closer to its *C*-terminus (Cys^{26} and Cys^{110} in β-hCG). It should be noted that the segment that forms the central part of the seat belt of the β-subunit (the region 93–100 in β-hCG) determines the specificity of the interaction of gonadotropin with LHCGR. It is important that in β-LH and β-hCG this segment has a net positive charge, which determines its interaction with the negatively charged orthosteric site LHCGR [79,81]. The *C*-terminal segment 88–92 of the α-subunit of hCG is also involved in binding to LHCGR [82,83].

Both α- and β-subunits of gonadotropins undergo *N*-glycosylation because they contain asparagine-containing sites, targets for *N*-glycosyltransferases, with the consensus motifs Lys-Asn-(Val/Ile) or (Glu/Tyr)-Asn-His [18]. In the α-subunit, common to all gonadotropins, two such sites are localized (Asn^{52}, Asn^{78}), while in the β-subunit there are one (β-LH, Asn^{30}) or two (β-hCG, Asn^{13} and Asn^{30}) sites for *N*-glycosylation. The β-subunit of FSH, like β-hCG, also has two sites for *N*-glycosylation (Fig. 2). The *C*-terminal extension of β-hCG also has four sites for *O*-glycosylation, including the residues Ser^{121}, Ser^{127}, Ser^{132}, and Ser^{138} as targets. Sites that are modified by *N*-glycans are located in all three loops (L1–L3) of the α- and β-subunits [18,19,84,85] (Fig. 2). The degree of glycosylation, localization and structure of *N*-glycans (branching, charge, etc.) in α- and β-subunits make a significant contribution to the formation of gonadotropin heterodimeric complexes and their stability, and also determine the binding characteristics and effectiveness of gonadotropins, the bias of their signaling, and affect their pharmacokinetics [18,19,85,86] (see also Section 6).

A representative of a rather unusual group of gonadotropins with LH activity is equine chorionic gonadotropin (eCG), which is encoded by a single gene and combines the properties of LH and FSH when acting on the reproductive system of various (non-equid) mammals [87–90]. Unlike hCG, the β-subunit of eCG has only one site for *N*-glycosylation (Asn^{13}), but in the *C*-terminal region it contains up to 11–12 sites for *O*-glycosylation, and the targets of *O*-glycosyltransferases are serine (Ser^{118}, Ser^{123}, Ser^{128}, Ser^{130}, Ser^{137}, Ser^{140}, Ser^{141}, and Ser^{149}) and threonine residues (Thr^{127}, Thr^{129}, Thr^{131}, and Thr^{133}) [91] (Fig. 2). The degree of glycosylation of these sites ranges from 20 to 100%, and this is the reason that β-eCG is one of the most highly glycosylated proteins, in which the glycosyl component accounts for up to 40% of the molecular weight. Along with β-eCG, the horse has β-LH, which is also encoded by a single gene and has dual specificity, activating both gonadotropin receptors, LHCGR and FSHR. Like β-eCG, β-eLH has up to 11 sites for *O*-glycosylation, but is glycosylated to a lesser extent (the degree of glycosylation of these sites is from 10 to 77%) [87,91,92] (Fig. 2).

3. Structure of the Luteinizing Hormone/Chorionic Gonadotropin Receptor and the Mechanisms of Its Binding to Gonadotropins

The LHCGR belongs to the superfamily of serpentine receptors functionally coupled to heterotrimeric G proteins (GPCRs), and is included in the group δ of the rhodopsin family, together with FSHR, thyroid-stimulating hormone receptor (TSHR), and relaxin and insulin-like factor-3 (INSL3) receptors. Gonadotropins bind specifically to the high-affinity orthosteric site of LHCGR, which is formed by a large extracellular domain, similar to that observed in other receptors of the δ group.

The LHCGR ecdomain includes up to 360 AARs and contains two structural subdomains, the first of which includes 9 leucine-rich repeats (LRRs), while the second is a large hinge region. connecting the LRR subdomain to the TMD. The main structural elements of the TMD are seven hydrophobic transmembrane helices (TMs) that form the transmembrane tunnel. At the *N*- and *C*-termini of the hinge region there are two more LRR segments, LRR10 and LRR11, between which the α-helix Pro^{272}–Asn^{280} (hinge helix) and an extended hinge loop [28]. At the *C*-terminus of the hinge region, where the extracellular domain connects to the TMD, a small region of P10 (Phe^{350}–Tyr^{359}) is located. The hinge region finely regulates the binding of gonadotropins to the ectodomain and ensures the signal transduction generated by them to the TMD, largely determining the differences in LH- and hCG-induced intracellular signaling [28,93].

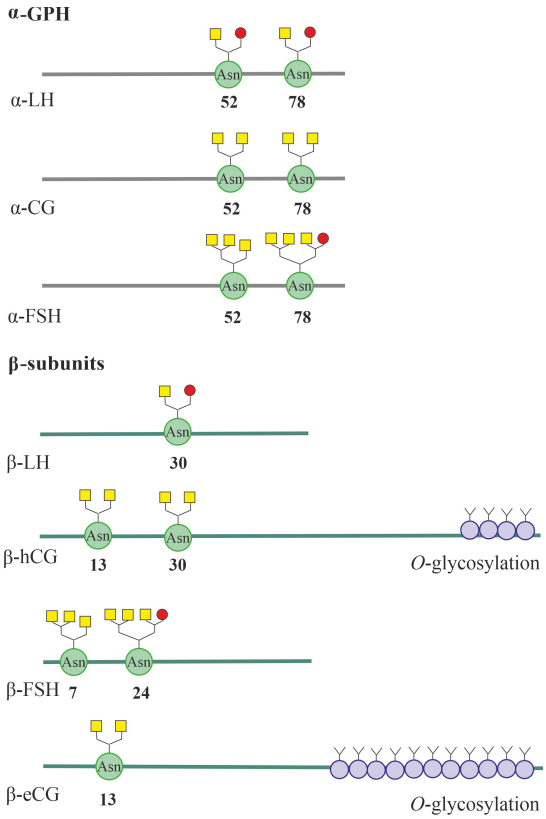


Fig. 2. The *N*- and *O*-glycosylation of the luteinizing hormone (LH) and human chorionic gonadotropin (hCG) subunits, as well as the follicle-stimulating hormone (FSH) subunits shown for comparison. For α-LH, α-FSH and α-CG, glycoforms of α-glycoprotein hormone (α-GPH) are presented, characteristic of the molecules LH, FSH and classical (placental) hCG. All gonadotropin subunits show sites for *N*-glycosylation, including asparagine residues. In the *C*-terminal part of the β-subunits of human and equine CG, sites for *O*-glycosylation, including serine and threonine residues, are also localized. The most typical structures of *N*-glycans characteristic of the presented gonadotropins are presented. In the α- and β-subunits of human and horse LH and CG, weakly branched (hybrid and bi-antennary) *N*-glycans predominate, and in LH secreted by the pituitary gland there is more terminal sulphated *N*-acetylgalactosamine (GalNAc), and in CG secreted by the fetus and placenta, sialic acid residues predominate. The α- and β-subunits of FSH contain a significant number of more branched (three- and four-antennary) *N*-glycans enriched in sialic acid residues. It should be noted, however, that under specific conditions (a certain phase of the estrous cycle, age, pathological conditions, etc.), *N*-glycans can differ significantly structurally, both between the same types of subunits and between the α- and β-subunits that form the dimeric gonadotropin complex. The terminal sialic acid residues are indicated by yellow squares, and the terminal sulphated *N*-acetylgalactosamine (GalNAc) residues are indicated by red circles. CG, chorionic gonadotropin; eCG, equine chorionic gonadotropin.

The surface of hCG, which is an $\alpha\beta$-heterodimer complex, contains clusters enriched with positively charged AARs that electrostatically interact with clusters of negatively charged AARs that form the ligand-binding surface of the LHCGR ectodomain. Using cryoelectron microscopy, it was shown that both subunits, the hCG-specific β-subunit and the α-subunit common to all glycoprotein hormones (α-GPH), are involved in the interaction with LHCGR. The *C*-terminal segment 92–106 of β-hCG specifically interacts with the residues Arg^{53}, Ser^{55}, Ala^{57} and Tyr^{58}, located in LRR1, and with the Glu^{206}, located in LRR7, and the residues Val^{46} and Gln^{48} of β-hCG form contacts, respectively, with the residues Gln^{246} and Arg^{247} located in LRR10. In turn, residues Tyr^{88}, Tyr^{89}, and Ser^{92} of α-GPH interact with residues Tyr^{127}, Ile^{152}, Lys^{180}, and Tyr^{182}, located in LHCGR repeats LRR4–LRR6 [28]. When hCG binds to the receptor, significant conformational changes are observed in four segments: in a region localized in β-hCG, called the "seat belt", which is responsible for stabilizing the $\alpha\beta$-heterodimer of hCG, as well as in β-sheet structures localized in β-hCG (L2, L3) and α-hCG (L3) [28,94]. Despite the similarity of the "seat belt" in the β-hCG and β-LH, there are significant differences between the β-sheet structures of these subunits, which entails significant differences in the efficiency of their interaction with LHCGR and in the ability to selectively activate intracellular signaling cascades, first all due to the different pattern of their interaction with the hinge region LHCGR [28,93].

As in the case of a number of other representatives of class A GPCRs, during gonadotropin-induced activation of LHCGR, a change in the superposition of the TM6 helix and the interacting TM5 and TM7 helices in the TMD occurs, despite the fact that gonadotropin binding occurs in the extracellular part of LHCGR. Changes in the relative position of TMs and the configuration of the internal cavity of the TMD are a trigger for conformational changes in the heterotrimeric G protein associated with the cytoplasmic regions of the receptor. These conformational changes promote the guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange in the Gα subunit of the G protein and weaken its association with the G$\beta\gamma$ dimer, which leads to the activation of Gα subunit and G$\beta\gamma$ dimer-dependent intracellular signaling cascades.

When hCG binds to LHCGR, the *C*-terminal segment of the TM6 moves outward (by 12.8 angstroms), and this is accompanied by a slight outward shift (by 2 angstroms) of the TM5 helix and an inward shift (by 3.6 angstroms) of the TM7 helix [28]. An assessment of the distance between different segments of the TM6 and TM7 by Xinheng He and his colleagues [95] using molecular dynamics showed that when LHCGR binds to hCG, the distance between them increases both in the outer vestibule of the transmembrane tunnel and in its central part, and this is accompanied by a significant increase in the volume of the internal cav-

ity of the transmembrane tunnel. As a result, in the hCG-bound LHCGR, the average distance between the α-carbon atoms of the $Ala^{592(6.58)}$ and $Lys^{605(7.35)}$ residues, localized in the extracellular ends of the TM6 and TM7, is 12.9 $\pm$ 1.3 angstroms, while in the hormone-unbound receptor it is significant in short, only 7.7 $\pm$ 3.5 angstroms. The distance between the α-carbon atoms of residues $Cys^{581(6.47)}$ and $Asn^{615(7.45)}$, located in the central part of the TM6 and TM7, in hCG-bound LHCGR is 8.5 $\pm$ 0.2 angstroms, which also, although to a small extent, exceeds this value in hormone-free receptor (7.2 $\pm$ 0.9 angstroms).

It should be noted that in the ternary complex of hCG–LHCGR–G_s protein, the distance between the TM6 and TM7 is similar to that in the double complex of hCG–LHCGR. It is important that the calculated volume of the internal cavity of the TMD for the double (253.7 $\pm$ 121.1 $Å^3$) and ternary (188.4 $\pm$ 111.3 $Å^3$) complexes significantly exceeds that for the hormone-free receptor (313.03 $\pm$ 162.09 $Å^3$) [95]. In this case, the entrance to the transmembrane tunnel expands to the greatest extent, which also occurs when small ligands bind to the TMD of a large number of GPCRs [96]. The result of an increase in the volume of the internal cavity of the TMD and associated changes in the superposition of its TMs is a change in the conformation of the cytoplasmic regions proximal to the membrane, belonging to its second and third ICLs (ICL2, ICL3) and the cytoplasmic C-terminal domain. These regions contain the main molecular determinants responsible for the interaction of LHCGR with various types of G proteins and β-arrestins.

The basis for changes in the superposition of TMs upon activation of LHCGR by gonadotropin is a change in the interaction between the LRR subdomain and the hinge region of the ectodomain, on the one hand, and the TMD, primarily the ECLs, forming the outer vestibule of the transmembrane tunnel, on the other [95]. In the absence of hormonal activation, the close interaction between the LRR subdomain and the TMD ensures that the latter remains in an inactive state. After binding to gonadotropin, the interaction of the LRR subdomain with the TMD is weakened, as indicated by a significant increase in the distance between them. In this case, the LRR subdomain moves into a vertical position relative to the TMD. In turn, the hinge region, on the contrary, as a result of its rotation, approaches the extracellular vestibule of the TMD, while simultaneously moving away from the LRR subdomain [97]. This is indicated by the results of assessing the mobility and structural changes in the LRR subdomain, hinge region and TMD of LHCGR during its transition from the active to the inactive state using molecular dynamics methods. Calculations show that upon activation of LHCGR, the distance between the LRR subdomain and the TMD can increase on average from 60 to 88 angstroms, while the distance between the hinge region and the TMD decreases from 79 to 48 angstroms [97]. Of key importance in the association and functional coupling of the ectodomain and the TMD of LHCGR are the interactions between the helix of the hinge region of the ectodomain and the helix formed by the middle part of ECL1, as well as the interactions between the *C*-terminal segment P10 of the hinge region, which borders the upper part of the TMD bundle, with one side, and the outer vestibule of the transmembrane tunnel formed by the extracellular ends of helices TM1, TM2 and TM7 and all three ECLs, on the other [28,95,98,99]. A change in the nature of these interactions upon binding of LHCGR to gonadotropin affects the relative position of the TM5, TM6 and TM7 helices and the conformation of the TMD as a whole, including its cytoplasmic regions, thereby activating intracellular signaling.

Among the molecular determinants located in the LHCGR ectodomain that mediate its activation by gonadotropin, the Tyr^{331} residue plays an extremely important role, which undergoes sulfation at the hydroxyl group in the phenolic ring and therefore acquires a negative charge. This residue is located in the middle of the hinge region and is surrounded by negatively charged AARs, thereby forming a cluster with a high negative charge density. The Tyr^{331} and its neighboring residues Asp^{330} and Glu^{332} electrostatically interact with positively charged hCG clusters, thereby controlling the relative position of the hinge region and the TMD of LHCGR. Substitutions of these residues with other AARs that disrupt the integrity of the anionic cluster prevent effective interaction with gonadotropin and lead to a significant decrease in its ability to activate LHCGR [100]. Another important determinant is Ser^{277}, which is involved in the formation of the Pro^{272}–Asn^{280} helix, localized in the *N*-terminal part of the hinge region. It mediates the interaction of helix 272–280 with the ECL1 helix and thereby modulates the stability of the complex between the ectodomain and the TMD. It is also suggested that the hydroxyl group of Ser^{277} is capable of forming a hydrogen bond with Asn^{351}, located in the highly conserved region P10 [28], which is often considered as a tethered agonist for LHCGR [98]. It should be noted that a structurally similar region is also localized in the corresponding TSHR locus, which also functions as a tethered agonist.

Along with the full-length forms of LHCGR, shortened soluble forms that lack the transmembrane domain are generated in men and women [101,102]. The blood level of the soluble LHCGR form negatively correlates with the success of embryo implantation and is one of the risk factors for premature birth and miscarriage [103]. Soluble LHCGR forms, localized in the cytosol, make a significant contribution to the malignancy of adrenal cells [104]. These forms of the LHCGR are capable of negatively influencing LH signaling by suppressing the effects of LH and hCG, including the formation of inactive heterodimeric complexes with protomers of the full-length LHCGR, and thus can be considered as negative allosteric LHCGR modulators [105]. In addition, the protomers of the soluble LHCGR form a complex with FSHR and prevent its translocation into the

plasma membrane, thereby reducing the number of surface FSHRs and weakening FSH-dependent responses [106].

4. Common Principles of Organization and Functioning of Gonadotropin-Regulated Signaling Cascades

4.1 Intracellular Signaling Cascades, Targets of LH and hCG

Specific binding of $\alpha\beta$-heterodimers of LH and hCG to the extracellular domain of LHCGR induces conformational rearrangements in it, which, as noted above, affect the hinge region and the ECL2 and ECL3 contacting this region. The relative position and conformational mobility of ECL2 and ECL3 directly affect the structure of the TMD and the interaction of ICL2 and especially ICL3 with the G proteins and β-arrestins. Using a multi-step mechanism of conformational rearrangements, gonadotropins regulate intracellular signaling cascades, both through the activation of various types of G proteins associated with LHCGR, and by recruiting adapter and regulatory proteins, including various isoforms of β-arrestins, into complex with the receptor. It is necessary to take into account the fact that LHCGRs embedded in the membrane are capable of forming both homodimeric (homooligomeric) complexes and heterodimeric (heterooligomeric) complexes with other GPCRs, including FSHR. This will have a significant impact on the molecular mechanisms and selectivity of signal transduction from the extracellular domain of LHCGR to intracellular effector proteins. Transactivation of LHCGR is also important. It consists in the fact that the hormone binds to one of the protomers of the di(oligo)meric receptor complex, while signal transduction to intracellular effectors occurs through another protomer, and the latter can be the protomer of another GPCR, for example, FSHR.

Two pathways play a decisive role in the implementation of gonadotropin signaling via LHCGR [12,14,15]. There are the cyclic adenosine monophosphate (cAMP)-dependent pathway, including LHCGR–G_s protein–adenylyl cyclase (AC)–cAMP–protein kinase A (PKA)/Exchange Protein directly activated by Cyclic AMP (EPAC) family factors, and the phospholipase pathway, including LHCGR–$G_{q/11}$ protein–phosphoinositide-specific phospholipase Cβ (PLCβ)–diacylglycerol (DAG)/inositol-3,4,5-triphosphate (IP3)–protein kinase C (PKC)/calcium signaling (Fig. 3). In cells of the reproductive system, they participate in the gonadotropin-induced regulation of steroidogenesis, in the control of proliferation, angiogenesis, apoptosis, autophagy and other fundamental cellular processes [12,14,15]. In this case, β-arrestin pathways, which have been intensively studied recently, also play a significant role. Through them, the cascade of mitogen-activated protein kinases (MAPKs) is activated, the regulation of which can also be carried out through a G protein-dependent pathway, including cAMP-activated PKA (Fig. 3). These signaling pathways themselves and their regulation by gonadotropins with LH activity are discussed below, with an emphasis on the differences in the signaling properties of LH and hCG.

Binding of LH or hCG to the receptor leads to its transition to an active conformation and triggering of several intracellular signaling cascades, which can be carried out both through various classes of heterotrimeric G proteins and through arrestins. After activation of the G_s protein, a free GTP-bound α_s subunit is formed, which mediates stimulation of the membrane-bound AC, and this leads to an increase in the level of intracellular cAMP and stimulation of PKA and/or factors of the EPAC family. Along with this, after the formation of the gonadotropin-receptor-G_s protein-AC complex, it is possible to form a signalosome that includes this complex, which is translocated into the cell, providing a targeted increase in the level of cAMP in its individual compartments. PKA phosphorylates a large number of intracellular proteins that have specific sites for PKA phosphorylation, including the transcription factor cAMP response element-binding protein (CREB), and this largely ensures the regulatory effects of gonadotropins on steroidogenesis, maturation of generative cells, as well as on metabolism, growth, and survival of cells of the reproductive system.

Gonadotropin-induced dissociation of $G_{q/11}$ protein leads to the generation of a free $\alpha_{q/11}$ subunit, which activates PLCβ, which catalyzes the formation of second messengers, DAG and IP3. DAG causes stimulation of DAG/phorbol-activated PKC isoforms and PKC-mediated phosphorylation of intracellular effector proteins, while IP3 leads to activation of calcium channels of the endoplasmic reticulum, causing the release of Ca^{2+} from intracellular stores and changes in the activity of many Ca^{2+}-dependent proteins, as well as activation of the MAPK cascade, primarily the extracellular signal-regulated kinases 1/2 (ERK1/2). Some PLC isoforms, PLCβ2 and PLCβ3, can also be activated by the $\beta\gamma$ dimer, the main donor of which is the G_i protein. In this case, the α_i subunit formed during the dissociation of this protein can have an inhibitory effect on gonadotropin-induced AC activation, preventing the overproduction of cAMP. In addition, a decrease in the level of cAMP occurs due to its hydrolysis by cAMP-specific phosphodiesterases, such as type 4 phosphodiesterase (PDE4), PDE6 and PDE7.

G protein-coupled receptor kinases (GRK)-mediated phosphorylation following hormonal activation of the LHCGR leads to the recruitment of β-arrestins, which not only inhibit G protein signaling, but also lead to the formation of early endosomes into which the ligand-receptor complex is included. Depending on the pattern of GRK phosphorylation, the resulting endosomes can either provide endosomal trafficking of such a complex with its subsequent lysosomal degradation or recycling of the LHCGR into the plasma membrane, or form a signalosome respon-

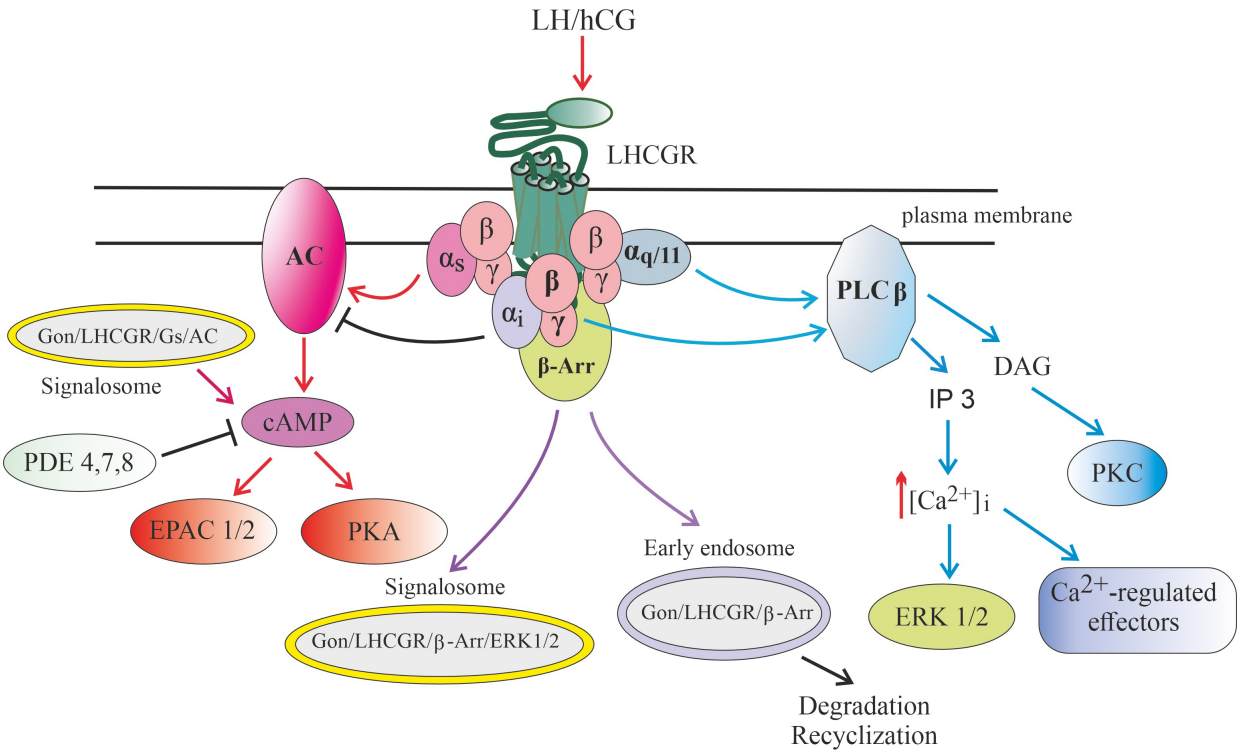


Fig. 3. Signaling pathways realized through luteinizing hormone/chorionic gonadotropin receptor (LHCGR), as well as endosomal signaling carried out with the participation of gonadotropin-bound LHCGR. *Abbreviation*: AC, adenylyl cyclase; cAMP, 3′-5′-cyclic adenosine monophoshate; β-Arr, β-arrestin; $[Ca^{2+}]_i$, intracellular concentration of calcium ions; DAG, diacylglycerol; EPAC1/2, Exchange Protein directly activated by Cyclic AMP, types 1 and 2; ERK1/2, extracellular signal-regulated kinases, types 1 and 2; α_s, α_i, $\alpha_{q/11}$, α subunits of $\alpha\beta\gamma$-heterotrimeric G_s, G_i and $G_{q/11}$ proteins, respectively; IP3, inositol-3,4,5-triphosphate; LHCGR, receptor of luteinizing hormone (LH) and chorionic gonadotropin (CG); PDE4,7,8, cAMP-activated phosphodiesterases, types 4, 7 and 8; PKA, protein kinase A; PKC, phorbol-sensitive protein kinase C, isoforms ε and δ; PLCβ, phosphoinositide-specific phospholipase Cβ.

sible for the activation of intracellular effectors, including ERK1/2, the effector components of the MAPK cascade.

4.2 cAMP-Dependent Signaling Pathways

Hormones, including LH and hCG, stimulate cAMP-dependent effector proteins and transcription factors through the "classical" AC signaling system, including as the main components GPCR, a heterotrimeric G_s protein consisting of a Gα_s subunit and a G$\beta\gamma$ dimer, and a membrane-bound isoform of AC, the catalytic component of this system. AC catalyzes the reaction of converting ATP into cAMP, which is a universal second messenger. After binding to the hormone, the conformation of the G protein-binding surface of the receptor changes, which includes segments of the ICLs and the intracellular vestibule of the tunnel formed by the TMD. This ensures effective interaction of the receptor with the G_s protein and its activation, including the replacement of GDP in the guanine nucleotide-binding site of the Gα_s subunit with GTP and the subsequent dissociation of the GTP-bound Gα_s subunit from the G$\beta\gamma$ dimer. The monomeric Gαs subunit in the active, GTP-bound state is capable of maintaining weak bonds with the G$\beta\gamma$ dimer, which allows it later, after hydrolysis of GTP and the transition of the Gα_s subunit to the inactive, GDP-bound state, to reassociate with G$\beta\gamma$ dimer at a high rate to form the inactive complex. The GTP-bound Gα_s subunit interacts with regulatory sites of AC, causing an increase in its catalytic activity and stimulating the production of intracellular cAMP.

Synthesized cAMP interacts with effector proteins specific to it, primarily with serine/threonine PKA and factors of the EPAC (Exchange Protein directly activated by Cyclic AMP) family, which are also called cAMP-regulated guanine nucleotide exchange factors exchange factor (cAMP-GEF) (Fig. 3). PKA in its inactive state is a heterotetrameric complex consisting of two regulatory (inhibitory) and two catalytic subunits. Each regulatory subunit includes two cAMP binding sites and two sites located in the *N*-terminal region that interact with the catalytic subunits [107]. When bound to cAMP, the regulatory subunits dissociate from the complex, the released catalytic subunits are activated and catalyze the transfer of the γ-phosphate group from ATP to the serine/threonine-containing site of the phosphorylated protein, the target of PKA. The main

targets of the enzyme are cAMP-regulated transcription factors, including the factor CREB (cAMP-responsive element binding protein), which controls the expression of many genes.

The EPAC family factors, Epac-1 (cAMP-GEF-I) and Epac-2 (cAMP-GEF-II), have one (Epac-1) or two (Epac-2) cAMP-binding sites in the *N*-terminal part, and in their *C*-terminal part there is a catalytic GEF domain, the function of which is to ensure GDP/GTP exchange and activation of small G proteins, such as Rap1 and Rap2 [108]. As in the case of PKA, one of the targets of the EPAC1 and EPAC2 is the transcription factor CREB, through which they regulate gene transcription, cell growth, apoptosis, cell migration, and mitochondrial dynamics [109]. It should be noted that, in addition to the activation of small G proteins, EPACs also activate a number of other effector proteins, including PLCϵ [110], Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) [111], phosphatidylinositol 3-kinase [112], and the components of the MAPK cascade [113,114]. All this significantly expands the range of their physiological effects in response to cAMP stimulation. Since the dissociation constants for the binding of cAMP to PKA and EPACs have similar values, in each specific case the choice of effector is determined not by the intensity of cAMP production, but by the availability of PKA and EPACs for activation by the cyclic nucleotide [115].

In addition to the effector components of cAMP signaling, phosphodiesterases (PDEs) play a significant and, in some cases, a decisive role in its regulation, causing the degradation of cyclic nucleotides, including cAMP, and thereby terminating the transduction of cAMP-dependent signals into the cell. The cells of the male and female reproductive system contain PDE4, PDE7 and PDE8, which are highly selective towards cAMP and hydrolyze it to inactive adenosine 5′-monophosphate (Fig. 3). Importantly, the expression and activity of these PDEs are characterized by cell and tissue specificity. In the ovaries, the PDE8A and PDE8B isoforms are found in significant quantities in theca cells and oocytes, the PDE4A isoform is found in oocytes and medullar stromal tissue, the PDE4C and PDE4D isoforms are found in follicles, the PDE7A and PDE7B isoforms are found in oocytes, while the PDE4B isoform is mainly in the outer layer of theca cells [116]. Dual-specificity PDEs that hydrolyze both cAMP and cyclic guanosine monophosphate (cGMP) may play a certain role in the control of cAMP signaling, for example, the ovarian isoform PDE3B. The hydrolytic activity of PDE7 and PDE8 is detected even at low concentrations of cAMP inside the cell, while PDE4 is activated only by relatively high concentrations of cAMP, under conditions of intense AC stimulation with gonadotropins [116]. Leydig cells have demonstrated high activity of the PDE8A and PDE8B isoforms and have been shown to be involved in the regulation and modulation of the steroidogenic effects of gonadotropins through cAMP-dependent pathways, and inhibition of these PDEs leads to increased signal transduction, similar to hormonal stimulation [117,118]. Thus, specific PDE inhibitors mimic the stimulating effect of gonadotropins with LH activity on target cells, which is of great practical importance for the pharmacology of reproductive disorders [116,118–122].

Phosphatases, which are targets for PKA and undergo phosphorylation, can also play a certain role in the transduction of the LH-induced signal. It has been shown that in ovarian granulosa cells the protein phosphatase 1 regulatory subunit 12A (PPP1R12A) and serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform (PPP2R5D) undergo cAMP-dependent phosphorylation, which leads to changes in the functional activity of the enzymes and causes dephosphorylation and inactivation of the receptor guanylate cyclase natriuretic peptide receptor 2 (NPR2), which is involved in the control of oocyte meiosis [123]. However, there is no reliable data on the direct influence of phosphatases on upstream components of cAMP signaling.

Important for cAMP signaling are scaffold proteins, which ensure the integration of GPCR, G proteins, AC, PKA, PDEs and other signaling and effector proteins into multicomponent molecular assemblies. Among scaffold proteins, proteins of the A-Kinase Anchoring Proteins (AKAP) family, which specifically interact with the regulatory subunit of PKA, play an important role in the control of cAMP signaling. This interaction includes the *N*-terminal helical domain of the PKA regulatory subunit, responsible for its dimerization and docking (*N*-terminal Dimerization/Docking domain), and the amphipathic α-helix of AKAP. These helical regions form a superhelical structure responsible for the retention of PKA in a particular cellular compartment and for the specificity of its interaction with target proteins [124]. Ezrin, radixin and moesin (ezrin-radixin-moesin, ERM), which are responsible for the formation and reorganization of the cytoskeleton and lipid rafts, participate in the regulation of the activity of EPACs. By specifically interacting with the *N*-terminal region of EPAC1, ERM proteins ensure the translocation of EPAC1 to the membrane and its inclusion in the signalosome, thereby redirecting cAMP signaling to the EPAC-dependent pathway [125].

4.3 Phospholipase Signaling Pathways

The result of hormonal activation of the $G_{q/11}$ protein, mediated through GPCRs, including LHCGR, is the exchange of guanine nucleotides in the α-subunit of the $G_{q/11}$ protein, the dissociation of the GTP-bound $G\alpha_{q/11}$ subunit from the $G\beta\gamma$ dimer and its functional interaction with phosphoinositide-specific PLCβ. Activated in this way, PLCβ hydrolyzes the membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2), generating two important second messengers, such as membrane-bound diacylglycerol (DAG) and water-soluble inositol-1,4,5-triphosphate (IP3).

DAG causes activation of phorbol-sensitive PKC isoforms, while IP3 mobilizes calcium ions from intracellular stores [126–128] (Fig. 3). The regulatory effects of IP3 are realized through its binding to IP3-specific receptors localized in the membrane of the endoplasmic reticulum [129]. This leads to the leakage of calcium ions from intracellular stores, an increase in their concentration in the near-membrane space of the endoplasmic reticulum and, as a consequence, to the activation of Ca^{2+}-activated ryanodine receptors, which have a high conductivity for Ca^{2+} [130]. A rapid increase in Ca^{2+} concentration inside the cell leads to the activation of a large number of calcium-regulated proteins, primarily Ca^{2+}-calmodulin-dependent, among which the most important are various isoforms of Ca^{2+}-calmodulin-dependent protein kinase II [127]. The rapid increase in Ca^{2+} concentration in certain cell compartments caused by IP3 is subsequently quickly eliminated by pumping Ca^{2+} out of the cytosol using both plasma membrane calcium channels and Ca^{2+}-ATPases localized in the sarcoplasmic reticulum membrane. This is due to the need to protect cells from hyperactivation of Ca^{2+}-dependent effector systems, which reduces cell survival. Termination of the $G_{q/11}$-mediated signal is also carried out by the elimination of DAG and IP3, which are recycled and, after phosphorylation, are converted back into PIP2, replenishing the reserves of this phosphoinositide for the subsequent signal transduction cycle [131,132]. Within just a few minutes after hydrolysis induced by activation of the $G_{q/11}$-mediated phospholipase pathway, rapid restoration of the PIP2 pool begins, despite continued exposure of the cell to the hormonal stimulus.

Back in the early 1990s, it was found that the hormone-activated $G_{q/11}$ protein, or more precisely its $G\alpha_{q/11}$ subunit, stimulates all four known isoforms of PLCβ (PLCβ1, PLCβ2, PLCβ3 and PLCβ4), while two of them, PLCβ2 and PLCβ3, can also be activated by the G$\beta\gamma$ dimer, the main donor of which is the pertussis toxin-sensitive $G_{i/o}$ proteins [133–135]. Since the generation of free G$\beta\gamma$ dimer occurs mainly due to the activation of $G_{i/o}$-coupled GPCRs, their agonists, like agonists of $G_{q/11}$-coupled GPCRs, can activate phospholipase pathways. Moreover, in the case of PLCβ2 and PLCβ3, a synergistic calcium response can be observed when agonists act simultaneously on $G_{q/11}$- and $G_{i/o}$-coupled GPCRs [136]. Another possibility is a synergism between the $G_{q/11}$- and $G_{i/o}$-mediated signaling pathways, which are activated by the hormone through the same GPCR, characterized by coupling to both types of G proteins, which is also true for LHCGR.

The target of the $G\alpha_{q/11}$ subunit and G$\beta\gamma$ dimer is the *C*-terminal domain of PLCβ, which, being in close interaction with the catalytic domain, inhibits the hydrolytic activity of PLCβ. In the case of the $G\alpha_{q/11}$ subunit, both the distal and proximal regions of the *C*-terminal domain are involved in binding to it, while in the case of the G$\beta\gamma$-dimer, only its distal regions, and the interaction of PLCβ with the G$\beta\gamma$ dimer induces more pronounced conformational changes in the *C*-terminal domain [137]. In both cases, binding of the *C*-terminal domain to the G protein prevents the inhibitory effect of this domain on the catalytic domain and ensures high levels of PLCβ activity.

4.4 β-Arrestin Signaling Pathways

Adapter proteins β-arrestins, which, like G proteins, are capable of specifically interacting with the membrane-proximal regions of the ICLs and the cytosol-oriented vestibule of the GPCR transmembrane tunnel, are present in all types of cells and tissues and are involved in the control of most physiological processes [138–141]. Of decisive importance for GPCR signaling, including that realized through LHCGR, are two forms of β-arrestins, such as βarr1 and βarr2, which are also designated as β-arrestins-2 and -3 (78% homology of the primary structure). βarr1 and βarr2 are cytosolic proteins, although βarr1 can be localized within the nucleus. Their main function is the ability to disrupt the interaction of ligand-activated GPCR with G protein and cause internalization of the ligand-receptor complex within the early endosome into the cell [142]. Along with this, β-arrestins form an active complex with GPCRs, which has its own signaling functions. This complex is responsible for the activation of a number of intracellular effectors, including ERK1/2 (extracellular signal-regulated kinases, types 1 and 2), the effector components of MAPK cascade [141]. Formation of an active complex with the receptor requires dephosphorylation of Ser^{412} (βarr1) or Thr^{383} (βarr2) located in the *C*-terminal part of β-arrestins, which are in a phosphorylated state before interacting with the ligand–GPCR–G protein complex [141,143]. It is important to note that dephosphorylation of β-arrestins is not necessary for β-arrestin-mediated GPCR desensitization that occurs upon receptor association with β-arrestins, since this process depends primarily on the pattern of β-arrestin isoforms and their relationship with receptors and other components of signal transduction.

β-Arrestin-mediated GPCR desensitization is due to the interaction of β-arrestin with receptor sites that, after hormonal activation, are phosphorylated either by GPCR-specific kinases (GRK, homologous desensitization) or by low-specific PKA and PKC (heterologous desensitization). However, in the cytoplasmic regions of GPCRs, as a rule, there are several target sites for phosphorylation by protein kinases, and the phosphorylation pattern depends on many factors. Among them are the structural features of the active, hormone-bound conformation of the receptor, the ability of the receptor to form homo- or hetero-oligomeric complexes, the type and ratio of heterotrimeric G proteins interacting with the receptor, as well as the type of protein kinases acting on the receptor, including various isoforms of GRKs, the action of which on GPCR sites, targets for phosphorylation, is characterized by high specificity.

The GPCR phosphorylation pattern is, in a certain sense, a "phosphocode" that determines further signaling events involving β-arrestins. Thus, the "phosphocode" programs the internalization and subsequent degradation or recycling of GPCRs, or favors the formation of a signalosome comprising the GPCR-β-arrestin complex and the initiation of β-arrestin-mediated signaling cascades.

Among the effector components of the MAPK cascade, the main target of β-arrestins is protein kinases of the ERK-family [141,144,145] (Fig. 3), and various mechanisms of their activation are possible. According to one of them, β-arrestins act as scaffold proteins that ensure colocalization and coordinated functioning of components of the MAPK cascade, such as protein kinases Raf1, MEK1 and ERK1/2 [145,146], and βarr1 and βarr2 differ in their influence on the activity of this cascade [141,147]. Along with this, there is evidence of the ability of β-arrestins, mainly βarr1, through other mechanisms to directly activate Raf kinase, an upstream component of the MAPK cascade [148], and a downstream effector protein, the proto-oncogenic kinase Src [149]. The binding of βarr1 to c-Raf kinase occurs in its Ras-binding domain, since in the presence of the small GTPase H-Ras, which also stimulates the activity of c-Raf kinase, the binding of the enzyme to β-arrestin is inhibited [148]. It has been established that β-arrestins can stimulate the activity of other members of the MAPK cascade, such as p38-MAPK and c-Jun N-terminal kinase, but no direct interaction between them has been detected [150,151].

A study of the molecular mechanisms of β-arrestin involvement in GPCR-mediated signal transduction using genetically modified cells lacking certain types of G proteins showed that β-arrestin signaling, including that mediating the activation of the MAPK cascade, requires the presence of functionally active heterotrimeric G proteins, at least in small quantities [152]. It is believed that the functions of β-arrestins consist of fine regulation and modulation of MAPK activity, which is based on their allosterically determined influence on the functional interaction of the hormone-activated receptor complex, including G proteins, with protein kinases ERK1/2 [153,154]. Thus, the question of the independence of β-arrestin signaling from G proteins has not been fully resolved, and the discussion here, most likely, should be about the coordinated interaction of G proteins and β-arrestins within the signalosome at the post-receptor stages of signal transduction.

5. Regulatory Effects of hCG and LH on Intracellular Signaling Cascades: Similarities and Differences

5.1 Stimulating Effects of LH and hCG on cAMP-Dependent Signaling Pathways

Back in the early 1990s, it was shown that both hormones, LH and hCG, despite their inherent structural differences, including different glycosylation patterns, are capable of stimulating the activity of membrane-bound AC isoforms and increasing the level of cAMP in frog oocytes and in cultured intestinal L-cells that express mouse LHCGR [155,156]. Gonadotropins also stimulated the phospholipase cascade and caused the mobilization of calcium ions from intracellular stores. Both processes were to a certain extent independent of each other, which indicated the absence of significant interaction between signaling cascades realized through heterotrimeric G_s and $G_{q/11}$ proteins, which are transducers between hormone-activated LHCGR and the effector proteins, AC and PLCβ respectively [155,156]. An increase in cAMP levels and a sharp rise in intracellular Ca^{2+} concentration after activation of LHCGR by gonadotropins occurred quite quickly, on average one minute after exposure, indicating similar kinetics of these processes [11,157]. Back in 1996, it was suggested that G_i proteins, with which ligand-activated LHCGR is also able to interact, may be involved both in the negative regulation of AC activity after its G_s-mediated stimulation by gonadotropin (short negative feedback) and also act as donors G$\beta\gamma$ dimer involved in the activation of PLCβ2 and PLCβ3, responsible for the regulation of calcium signaling and stimulation of various isoforms of PKC [158] (Fig. 3).

The half-maximal concentration (EC_{50}) for stimulation of AC activity and increase in intracellular cAMP levels by gonadotropins are in the picomolar range of their concentrations, while nanomolar concentrations of LH and hCG are required to trigger β-arrestin (βarr2)-mediated internalization of LHCGR [11]. Along with this, it was established that to achieve the maximum stimulating effect of gonadotropins on cAMP-dependent cascades, occupancy of no more than 10% of LHCGR is sufficient, while for effective translocation of β-arrestins to the ligand-receptor complex and its further internalization, the degree of occupancy of LHCGR should have exceeded 90% [11].

Activation of AC by gonadotropins with LH activity leads to an increase in the level of phosphorylation of the transcription factor CREB and stimulation of CREB-dependent expression of the gene encoding the cholesterol-transporting Steroidogenic Acute Regulatory protein (StAR), which catalyzes the first, rate-limiting stage of steroidogenesis, as demonstrated in Leydig cells [12]. Thus, the mechanisms of the influence of LH and hCG on AC activity and cAMP-dependent intracellular targets are characterized by a certain similarity. At the same time, the effectiveness of such influence varies quite significantly [10,12,14].

Most of the data obtained using cell cultures demonstrate a higher potential of hCG, compared to LH, regarding the activation of the AC system and downstream effector units (PKA) (Table 2). In cultured COS-7 cells expressing LHCGR, the ED_{50} value for the stimulatory effect of hCG on cAMP production was shown to be 107 $\pm$ 14 pmol/L, while the corresponding value for LH was five

Table 2. Comparison of the effects of luteinizing hormone (LH) and human chorionic gonadotropin (hCG) on adenylyl cyclase activity and cyclic adenosine monophosphate (cAMP)-dependent pathways—similarities and differences.

Similarities
Both gonadotropins in picomolar concentrations stimulate the gonadotropin–LHCGR–G_s protein–AC–cAMP–PKA/EPAC1/2 system, resulting in stimulation of the activity of cAMP-dependent transcription factors, including CREB, and stimulation of steroidogenesis and other cAMP-dependent processes. Gonadotropins exert their effects with high efficiency both in ovarian cells (theca and granulosa cells) and in testicular Leydig cells.
Differences
(1) The stimulating effect of hCG on AC and cAMP production is achieved at lower concentrations and is more stable over time than that of LH, and this difference is specific to certain cell types and is most pronounced in granulosa cells, where the AC stimulating effect of LH is weakly expressed. The result is a more pronounced stimulatory effect of hCG at the early stages of steroidogenesis, despite the fact that at the later stages of steroidogenesis the effects of the hormones become comparable. (2) The stimulatory effect of hCG on the activity of ERK1/2, an effector component of the MAPK cascade, realized via cAMP-dependent pathways and PKA is more pronounced than that of LH. This, along with differences in the influence of LH and hCG on the expression of proteins that regulate apoptosis, determines that hCG preferentially activates pro-apoptotic cascades, and LH anti-apoptotic cascades. (3) LH and hCG have different effects on cAMP-dependent cascades during heterodimerization of LHCGR with FSHR. In the case of hCG, there is a potentiation of the AC stimulating effect, and in the case of LH this effect does not change significantly.

Note: AC, adenylate cyclase; CREB, cAMP-dependent transcription factor (cAMP Response Element-Binding protein); EPAC1/2, Exchange Protein directly activated by Cyclic AMP, types 1 and 2; ERK1/2, extracellular signal-regulated kinases, types 1 and 2, the effector components of mitogen-activated protein kinase cascade; FSHR, follicle-stimulating hormone receptor; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; MAPKs, mitogen-activated protein kinases; PKA, protein kinase A.

times higher at 530 $\pm$ 51 pmol/L [9]. When using hCG, the maximum stimulating effect on intracellular cAMP levels was achieved one hour or more after treatment, while when using LH this occurred faster, after 10 min [9]. The efficiency of increasing cAMP levels in HEK-293 cells with LHCGR expressed in them when exposed to hCG was significantly higher than when exposed to LH, and the EC_{50} value for hCG was 213 pmol/L and was significantly lower than that for LH (426 pmol/L), with the maximum response for both gonadotropins being achieved at a concentration of about 10 nmol/L [17]. The differences in the effectiveness of LH and hCG were greatest in ovarian granulosa cells [14]. Treatment of a primary culture of human granulosa cells (hGLC) for 36 hours with hCG caused a more pronounced and time-sustained increase in cAMP levels compared to treatment with recombinant LH taken at an equivalent dose. As a result, in the culture treated with hCG, progesterone production was significantly higher than in the control and in the culture treated with LH, indicating a more pronounced stimulation of ovarian steroidogenesis caused by hCG [9].

Similar results were obtained with long-term treatment of cultured goat ovarian granulosa cells with gonadotropins, where hCG increased the intracellular level of cAMP and activated PKA with significantly greater efficiency than LH [159]. The stimulating effect of hCG on cAMP levels and cAMP-dependent phosphorylation of ERK1/2 during treatment of primary Leydig cell culture also significantly exceeded the corresponding effects of LH, in the case of stimulation of intracellular cAMP production by almost 10 times [12]. The higher potential of recombinant hCG for AC activation, compared with recombinant LH, was demonstrated in mouse Leydig tumor cells (mLTC-1), in which mouse LHCGR was expressed, as well as in HEK273 cells expressing human LHCGR [13]. This indicates that the peculiarities of obtaining recombinant forms of gonadotropins do not significantly affect the higher potential for stimulation of the AC system detected in the case of hCG, since both urinary and recombinant hCG were equally superior to LH.

It should be noted, however, that cAMP level-dependent indicators such as the degree of phosphorylation of the CREB factor and the expression of the gene for the steroidogenic protein StAR in cultures of Leydig cells and HEK273 cells treated with hCG and LH did not differ significantly [12,14]. This may be due to counter-regulatory influences that are triggered in the target cell with a long-term and sustained increase in cAMP concentration, among which an increase in the activity of cAMP-specific PDEs plays a significant role. It is also interesting that progesterone production in different cell types treated with hCG was significantly higher than with LH treatment, while testosterone levels varied slightly [13]. Laura Riccetti *et al.* [13] explain this disappearance of differences in the testosterone levels by the fact that in the case of exposure to LH, the synthesis of testosterone is carried out to a greater extent by the pathway that includes 17-hydroxyprogesterone as an intermediate instead of progesterone (the so-called Δ5-pathway), while hCG ensures the synthesis of large quantities of progesterone, which functions as a "parallel accumulation product" and then largely enters the less efficient Δ4 pathway of androgen synthesis. The result of this is a leveling off of testosterone production induced by both gonadotropins during later stages of testicular steroidogen-

esis, despite significantly greater amounts of progesterone accumulating in hCG-treated cells [13].

A study of the effects of hCG and LH (100 pmol/L) in human granulosa-lutein cells revealed that hCG not only stimulates phosphorylation of the factor CREB and increases the expression of the StAR protein gene with greater efficiency compared to LH, but also enhances proapoptotic cascades to a greater extent [160]. The proapoptotic effect of hCG was strongly attenuated in the presence of physiological doses of β-estradiol (200 pg/mL), as illustrated by increased activity of the anti-apoptotic enzyme Akt kinase, assessed by its Ser^{478} phosphorylation, and inhibition of the cleavage of procaspase-3, a key component of the apoptotic pathway, leading to increased cell survival. With regard to the expression of the aromatase (cytochrome CYP19A1) gene, the situation is the opposite, and the stimulating effect of LH 72 hours after treatment exceeded that of hCG. This causes an increase in aromatase activity and stimulates the conversion of androgens into estrogens, which are necessary to maintain the growth of follicles and oocytes and increase the survival of granulosa-luteal cells [160]. LH also more effectively, compared to hCG, increased the expression of genes encoding the anti-apoptotic protein XIAP (X-linked inhibitor of apoptosis protein) and cyclin D2, thereby exerting a pronounced anti-apoptotic effect [160]. This indicates that LH-stimulated cAMP-dependent pathways are characterized by a greater anti-apoptotic potential, while the corresponding hCG-activated pathways are primarily triggers of proapoptotic cascades, although the resulting effect of gonadotropins on apoptosis is thought to depend on from many factors and counter-regulatory influences (Table 2).

Since testing for LH and hCG is carried out in both human and rodent cell lines and uses gonadotropins from different sources, it is important to evaluate the possible species-specific effects of their effects on cAMP-dependent pathways. With various combinations of gonadotropins and target cells, their primary effect, which consists of activation of the AC signaling system, in the case of hCG, as a rule, significantly exceeds that of LH, and this is due to differences in the efficiency of interaction of the ligand (LH or hCG) with the LHCGR ectodomain and the peculiarities of conformational changes caused in the TMD and the LHCGR–G_s protein interface due to such interaction [12–14,160–162]. However, at later, effector, stages of hormonal signal implementation, at the stage of phosphorylation of the factor CREB and the final stages of steroidogenesis, the differences in the effectiveness of LH and hCG begin to weaken and, ultimately, may disappear altogether. It should be noted, however, that this applies most to the stimulation of testicular steroidogenesis, while in follicular cells the differences between LH and hCG tend to persist into the later, effector stages of gonadotropin signaling [12]. To a certain extent, the equivalence of LH and hCG in terms of stimulation of testicular steroidogenesis is important for the treatment of androgen deficiency and hypogonadotropic hypogonadism in men with gonadotropins. Thus, there is an observation that treatment of a man with central hypogonadism sequentially with LH and hCG normalized his blood testosterone level to the same extent [163].

An important point is that the AC-stimulating effects of hCG and LH may be modulated differently in the presence of FSH. This is due to both the heterodi(oligo)merization of LHCGR and the structurally similar FSHR, which changes the efficiency and pattern of their activation by gonadotropins, and the cross-interaction of intracellular signaling cascades activated by FSH and gonadotropins with LH activity inside the target cell. Under the *in vitro* conditions using human granulosa-luteal cells, it was shown that in the presence of FSH, the ability of hCG to enhance the production of intracellular cAMP increases on average five times, which entails increased phosphorylation of the factor CREB and the steroidogenic response to hCG [164]. In this case, the AC stimulating effect of recombinant LH in the presence of FSH does not change significantly, as a result of which treatment of cells with a combination of recombinant LH and FSH does not lead to a significant increase in the level of CREB phosphorylation and the production of steroid hormones (Table 2). Interestingly, FSH potentiates the stimulatory effects of LH (not hCG) on the activity of ERK1/2, a key effector component of the MAPK cascade, and on the activity of Akt kinase, an important mediator of anti-apoptotic processes, which leads to increased cell growth and survival [164]. The effect of FSH on hCG- and LH-induced signaling is in a good agreement with the powerful proliferative potential of endogenous LH in the follicular phase and after trophoblast formation, as well as with the strongly pronounced steroidogenic effect of classical hCG, necessary to maintain pregnancy after leaving the luteal phase.

The participation of LH-stimulated cAMP-dependent mechanisms in the positive control of proliferation was shown for a pool of primordial follicles in the ovaries of prepubertal female mice treated with the anticancer drug cis-platinum, which blocks cell growth [165]. Simultaneous administration of LH and cis-platinum to mice preserved the activity of cAMP-dependent signaling pathways and prevented a decrease in the reserve of primordial follicles, maintaining the fertility of animals when they reached reproductive age [165]. At the same time, it cannot be excluded that the different effectiveness and pattern of activation of the effector components of the cAMP-dependent pathways caused by LH and hCG may differently influence the proliferative response of follicular cells to these gonadotropins.

It is a well-known fact that with long-term exposure of target cells to gonadotropins, there is a significant decrease in the density of LHCGR on the cell surface, which is based on the internalization of ligand-receptor complexes as part of early endosomes into intracellular compartments. A factor that accelerates the internalization of LHCGR is the

formation of large LHCGR-containing aggregates [166]. Using fluorescence microscopy in the early 2000s, it was shown that such aggregates are formed much faster and in larger quantities when LHCGR binds to hCG than when they bind to LH, and this causes a higher rate and intensity of desensitization and downregulation of LHCGR when they are activated by hCG [167,168]. Further studies showed that after hCG-induced activation, LHCGR accumulate in the form of significant aggregates inside the cell, both in detergent-resistant membrane microdomains with an average diameter of about 170 µm, and in smaller membrane structures with an average diameter of 80 to 160 µm. In cells without hCG treatment, a significant portion of LHCGR was localized in larger membranous structures (with an average diameter of about 200 µm), which were able to efficiently integrate into the plasma membrane. As soon as hCG was removed from the medium, large aggregates began to gradually dissociate and the released LHCGR moved into larger membrane vesicles, thereby acquiring the ability to effectively translocate into the plasma membrane and bind to the hormone [169]. LH with less intensity induced the formation of large aggregates containing LHCGR, which led to a less pronounced decrease in the sensitivity of target cells to gonadotropins with LH activity and to a more rapid recovery after LHCGR desensitization caused by prolonged exposure to gonadotropin (Table 2). The ability of hCG with greater intensity, as compared to LH, to induce the formation of large receptor aggregates is associated with a more effective interaction of the hCG–LHCGR complex with β-arrestins, which mediates the internalization of the ligand-receptor complex with subsequent aggregation of LHCGR and their movement into relatively small membrane microdomains [13].

5.2 Intracellular cAMP Signaling Activated by Gonadotropins with LH Activity

In the recent years, the paradigm that the internalization of GPCRs into the cell leads to the termination of hormonal signal transduction has undergone changes, since a lot of evidence has accumulated that, being part of the early endosome, ligand-receptor complexes continue to carry out their signaling functions already inside the cell. This is in good agreement with the localization of other components of cAMP signaling, primarily PKA and various PDE isoforms, in intracellular compartments, including the nucleus [170–172]. Moreover, it is assumed that for many receptors the main contribution to the regulation of the activity of effector proteins is made by "intracellular" signaling [173]. This is also quite applicable for cAMP signaling mediated through gonadotropin-stimulated LHCGR [174].

There is evidence that cAMP signaling for many types of GPCRs, including gonadotropin receptors, involves two phases of activation of its components, and these phases are separated both in space and time [175–178]. The first, fast phase includes signaling events that occur immediately after the hormone binds to the receptor and occurs in the plasma membrane, where functional coupling occurs between the ligand-activated GPCR, G_s protein and AC. It must be emphasized that previously all ideas about the functioning of the hormone-sensitive AC system were limited mainly to this phase, and further events were considered exclusively in terms of down-regulation of the ligand-activated receptor complex during endocytosis. However, following the fast phase, at least in some cases, there is a constant, longer phase of cAMP signaling called the endosomal phase. It starts after the internalization of the ligand-receptor complex as part of the early endosome into intracellular compartments, where cAMP synthesis occurs, and the formation of this second messenger can be spatially linked to a specific cell compartment [175,178,179]. This makes it possible to influence cAMP-sensitive effector proteins in specific compartments without inducing a total increase in the level of cAMP in the cell, which is intended to ensure the specificity and targeting of signal transduction.

Initially, it was believed that the implementation of the endosomal phase requires the interaction of hormone-activated G_s-coupled GPCR with β-arrestin, which ensures both the internalization of the signaling complex within the endosome and its functional activity in the second phase of cAMP signaling. However, it was later shown that this process may be independent of β-arrestins [178]. The function of β-arrestins in this case is only to delimit the fast phase of signal transduction, interrupting signal transduction through the G_s protein in the plasma membrane, from the late, endosomal phase. In this case, in the endosome, β-arrestins are no longer involved in signaling events. It is assumed that, depending on the phosphorylation pattern of the ligand-activated GPCR, β-arrestins act as "switchers", redirecting some ligand–GPCR–G_s protein complexes along the path of their degradation or recycling, and "packaging" other complexes into the forming signalosomes, in which intracellular endosomal cAMP signaling occurs [178].

For LHCGR, endosomal cAMP signaling was studied in detail using fluorescence resonance energy transfer (FRET) microscopy in intact follicles isolated from the ovaries of transgenic mice embedded with sensors to assess cAMP production [174]. Follicles were stimulated with LH (20 µg/mL) for 10 min, taking into account the fact that this time was sufficient for the internalization of LHCGR, after which the hormone was washed off. AC stimulation and increase in cAMP levels continued for at least 20 min [174]. This is in good agreement with the fact that lysosomal degradation of LHCGR occurs approximately 30–60 min after the onset of their activation by the hormone [180]. It can be assumed that such degradation is intended to stop endosomal cAMP signaling, which lasts significantly longer than it should if only its first, fast phase was implemented, which is inevitably followed by desensitization of LHCGR. In the presence of dynasor, an inhibitor of clathrin-mediated endocytosis, disruption of the second,

endosomal phase of cAMP signaling was detected [174]. It is important that a number of regulatory effects of LH are realized during long-term stimulation of cAMP-dependent effector proteins, which may be due to endosomal signaling.

5.3 Effects of Gonadotropins on cAMP Signaling in the Oocyte Mediated through Components of the cGMP System

Along with the direct influence of gonadotropins with LH activity on the level of cAMP in ovarian cells, their indirect, modulatory effects on cAMP signaling are possible without triggering the LHCGR–G_s protein–AC–cAMP signaling pathway, in which ligand-activated LHCGR and changes in intracellular levels cAMP is realized in different cellular compartments and in different cells, as well as at different time points. This mechanism is realized in the case of LH-dependent control of oocyte maturation. In this case, LH, acting on ovarian granulosa cells, regulates the activity of PDE3 in the oocyte, changing the ratio of the concentrations of cGMP and cAMP in it and, thereby, controls the functional activity of transcription factors responsible for the entry of oocytes into meiosis [181–183].

In the absence of an ovulatory LH surge, FSH, through activation of FSHR in granulosa cells, stimulates AC activity and thereby increases cAMP levels in these cells. Along with this, the expression of natriuretic peptide precursor C (NPPC) increases in granulosa cells, which subsequently binds to the receptor guanylate cyclase NPR2 on the surface of cumulus cells, causing the accumulation of cGMP in them. One of the factors activating the synthesis of cGMP is FSH-mediated stimulation of estrogen receptors by increasing the production of estradiol. In this case, NPPC functions as an autocrine factor, dependent on estrogen levels and controlling the ratio of cAMP- and cGMP-dependent pathways in the cumulus-oocyte complex. cGMP, like cAMP, enters the oocyte from cumulus cells through connexin-37 localized in gap junctions and, through a competitive mechanism, inhibits the activity of PDE3 [184]. Since this isoform of PDE hydrolyzes cAMP, cGMP-induced suppression of its hydrolytic activity causes an increase in the level of cAMP in the oocyte. It should be noted that part of cAMP enters the oocyte from granulosa cells through the connexin system, and part is synthesized directly in the oocyte using the constitutively active G Protein-Coupled Receptor 3 (GPR3) and G Protein-Coupled Receptor 12 (GPR12) functionally coupled to AC [181,185]. Elevated cAMP levels lead to activation of PKA, which phosphorylates and inhibits maturation promoting factor (MPF) composed of cyclin-dependent kinase 1 (CDK1) and cyclin b1. The result of this is the arrest of phase I of oocyte meiosis, which prevents its maturation [183]. Knockout of the genes encoding PDE3 and the GPR3 in the oocyte leads to a decrease in the level of cAMP and stimulates oocyte maturation [186], while PDE3 inhibitors [187–189], as well as positive modulators of cAMP signaling [190] cause the opposite effect, preventing oocyte meiosis. The relationship between an increase in the cAMP level in the oocyte and blocking phase I of meiosis is observed in oocytes of humans and various mammalian species, indicating the universality of this mechanism in the control of oocyte maturation.

The LH surge, through the activation of LHCGR in granulosa cells, leads to the suppression of the activity of intracellular androgen and estrogen receptors in them, thereby inhibiting the expression and secretion of NPPC and preventing its activating effect on NPR2 receptors in cumulus cells [191]. Along with this, activation of the LH cascade causes an increase in the expression of epidermal growth factor (EGF), which leads to activation of EGF receptors and a resulting increase in intracellular Ca^{2+} levels, which also contributes to the inhibition of NPR2 activity. It should be noted that stimulation of EGF-dependent pathways mediated by LH and hCG has been demonstrated in humans and a large number of experimental animals [181,192–194]. In addition to the suppression of cGMP synthesis, the LH surge leads to the activation of ERK1/2-family kinases, which prevents the entry of cyclic nucleotides, cGMP and cAMP, into the oocyte through the connexin gap junction system. Thus, the level of cGMP in the oocyte sharply decreases, which leads to activation of PDE3, a decrease in the intracellular concentration of cAMP and inhibition of PKA [181,183]. The result of a decrease in PKA activity is the abolition of the inhibitory effect of this enzyme on the MPF factor, which leads to stimulation of the activity of meiosis-associated proteins and ensures the resumption of oocyte meiosis.

Despite the fact that stimulation of EGF-dependent pathways by gonadotropins with LH activity and cross-talk between the signaling pathways stimulated by these gonadotropins and EGF is shown for both LH and hCG, differences in the influence of LH and hCG on oocyte meiosis cannot be excluded, and this may have great implications for ARTs and requires further research.

5.4 Effect of hCG and LH on Phospholipase Pathways and Intracellular Calcium Signaling

Both in the testes and ovaries, phospholipase signaling pathways, realized through the functional coupling of LHCGR with the $G_{q/11}$ proteins, play no less important role in LH-mediated signaling than cAMP-dependent pathways [15]. The $G_{q/11}$-mediated activation of PLCβ is required for normal ovulation, ensuring proper activation of progesterone receptors and follicle rupture [195,196] (Table 3). Stimulation of PLCβ activity through other $G_{q/11}$-coupled GPCRs may also contribute to the ovulatory response, as demonstrated for the prostanoid receptor. Activation of this receptor by prostaglandin F2α in luteal cells stimulates the expression of the *Akr1c18* gene, encoding 20α-hydroxysteroid dehydrogenase, which causes increased

Table 3. Comparison of the effects of luteinizing hormone (LH) and human chorionic gonadotropin (hCG) on phospholipase pathways – similarities and differences.

Similarities
Both gonadotropins stimulate the gonadotropin–LHCGR–$G_{q/11}$ protein–PLCβ–IP3/DAG–increased $[Ca^{2+}]_i$/phorbol-sensitive PKCs system, and this requires higher concentrations of gonadotropins than in the case of AC activation. LH/hCG-stimulated phospholipase pathways are involved both in the steroidogenic response and in the regulation of growth and survival of target cells.
Differences
(1) The effects of hCG on phospholipase pathways and calcium signaling are less pronounced compared to LH and have differences in the pattern of regulation. In addition, the steroidogenic effect of hCG is highly dependent on extracellular Ca^{2+} concentration, indicating a significant contribution of plasma membrane calcium channels to the hCG-induced increase in intracellular calcium levels, in addition to that of PLCβ activation.
(2) LH activates ERK1/2 through $G_{q/11}$-mediated stimulation of phorbol-sensitive PKC, and thereby has a pronounced proliferative effect on ovarian granulosa cells, while hCG is ineffective in this regard.

Note: AC, adenylate cyclase; DAG, diacylglycerol; ERK1/2, extracellular signal-regulated kinases, types 1 and 2; IP3, inositol-3,4,5-triphosphate; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; PKC, protein kinase C; PLCβ, phosphoinositide-specific phospholipase Cβ.

conversion of progesterone to 20α-hydroxyprogesterone and is necessary for normal parturition [196]. At the same time, LH-induced $G_{q/11}$-mediated signaling is not involved in the formation of the corpus luteum and maintenance of pregnancy, since deletion of the gene encoding the $G\alpha_{q/11}$ subunit in granulosa cells does not lead to disruption of these processes. Processes important for ovulation, such as the resumption of meiosis, proliferation of the cumulus cell layer and ovarian angiogenesis, dependent on the activity of the VEGF signaling pathways, are mediated mainly through LH-activated cAMP-dependent pathways and occur normally in granulosa cells deficient in genes encoding $G\alpha_{q/11}$ subunits, that is, they do not directly depend on phospholipase signaling [195,196].

In contrast to cAMP signaling, activation of phospholipase pathways requires significantly higher concentrations of gonadotropins, since, for example, the EC_{50} for hCG, which ensures activation of PLCβ and IP3-mediated mobilization of calcium ions from intracellular stores, is 20 times higher than the corresponding EC_{50} values for the same gonadotropin required for half-maximal activation of AC and an increase in the intracellular cAMP concentration [8]. The stimulating effect of hCG on the accumulation of IP3 in the target cell is more pronounced at high density of LHCGR and decreases at low density, which indirectly indicates the preference for the interaction of hormone-activated LHCGR with G_s rather than with $G_{q/11}$ protein. However, the EC_{50} value for hCG-induced stimulation of IP3 production at both low and high receptor densities remains unchanged [8]. The effectiveness of the stimulating effect of hCG on the mobilization of intracellular Ca^{2+} 60 seconds after treatment with gonadotropin was significantly higher than that for LH, but over time the differences disappeared, which was due to the activation of the processes of pumping Ca^{2+} from the cytosol. As a consequence, the integrated values for the increase in cytosolic Ca^{2+} concentration over a three-minute interval varied to a significantly lesser extent [17].

It is necessary to distinguish mechanisms caused by activation of PLCβ signaling pathways from mechanisms involving activation of L-type plasma membrane calcium channels, which make a significant contribution to the steroidogenic effects of gonadotropins. In this case, the entry of calcium ions into the cell is necessary to replenish their reserves in intracellular depots. The first data on the participation of plasma membrane calcium channels in the stimulatory effect of gonadotropins with LH activity on the expression of steroidogenic proteins and the production of steroid hormones were obtained in experiments on mouse Leydig tumor cells in 1999 [197]. It was shown that in a Ca^{2+}-enriched environment (Ca^{2+} concentration was 1.5 mM), the stimulating effect of hCG on the expression of the gene encoding the StAR protein is enhanced by 1.7 times, and this is associated with a significant increase in progesterone production. At the same time, when complexing agents that specifically bind calcium ions or the calcium channel blocker veropamil were added to the extracellular environment, a sharp decrease in hCG-induced stimulation of steroidogenesis occurred. The transcription steroidogenic factor-1 (SF-1) plays a positive role in the Ca^{2+}-induced potentiation of the steroidogenic effect of hCG, while the nuclear receptor DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) plays a negative role [197]. Subsequently, the role of L-type calcium channels in the modulation of gonadotropin-stimulated steroidogenesis was demonstrated in rat granulosa cells, where the inhibitory effect of amphetamine, which reduces the concentration of intracellu-

lar Ca^{2+}, both basal and increased under the influence of various agents, on gonadotropin-stimulated expression of steroidogenic genes was demonstrated [198].

Intracellular calcium signaling is involved not only in the steroidogenic effects of LH and hCG, but also in the implementation of their proliferative effects (Table 3), as demonstrated for LH in its action on the epithelial ovarian cancer cells OV207 and OVCAR-3 [199]. The basis of the proliferative effects of LH is the activation of the MAPK cascade, including its effector component ERK1/2, and LH-induced stimulation of ERK1/2 occurs both through activation of L-type plasma membrane calcium channels and through stimulation of the phospholipase pathway, the effector component of which is a DAG-sensitive protein kinase Cδ. LH-induced migration and proliferation of cultured ovarian cancer cells was suppressed by inhibiting the ERK1/2 stimulating effect of gonadotropin, which can be achieved by reducing the extracellular Ca^{2+} concentration using chelating agents, as well as inhibiting calcium current across the plasma membrane using the calcium channel blocker verapamil and by suppressing release of Ca^{2+} from intracellular stores by dantrolene. In addition, the stimulatory effects of LH on ERK1/2 were suppressed in the presence of protein kinase Cδ inhibitors, the nonselective GF 109203X and the highly selective rottlerin, as well as small interfering RNA specific for this enzyme [199]. It was found that cAMP-dependent signaling pathways, through which phosphorylation and activation of ERK1/2 can also occur, are not involved in this process [199].

Since hCG activates cAMP-dependent pathways with much higher efficiency, it is not surprising that it affects phospholipase pathways and ERK1/2 activity with significantly less efficiency and, accordingly, has a low proliferative potential and, moreover, even has antiproliferative activity. This is supported by the results of a comparative study of long-term exposure of a primary culture of goat granulosa cells to LH and hCG [159]. It was found that LH significantly stimulates the activity of DAG-sensitive protein kinase C and significantly increases the ratio of phosphorylated and non-phosphorylated forms of ERK1/2, which is associated with activation of ERK1/2-mediated proliferation of follicular cells, while hCG does not affect these indicators and, on the contrary, reduces the proliferation of granulosa cells, acting through cAMP-dependent mechanisms [159]. These data indicate a different pattern of effects of LH and hCG on phospholipase pathways and intracellular calcium signaling involved in the control of cell growth and differentiation (Table 3).

5.5 hCG- and LH-Induced Interaction of the LH/hCG Receptor with β-Arrestins

After activation of LHCGR by gonadotropin, which induces its functional interaction with G proteins, the process of recruitment of β-arrestins is triggered, resulting in the internalization of the ligand-receptor complex as part of early endosomes into the cell, and this leads either to the degradation of LHCGR or to the induction of intracellular signaling [200]. It is important that the mechanisms of interaction with β-arrestins for LHCGR differ significantly from those in the case of FSHR. Recruitment of β-arrestins and the formation of their complex with hormone-activated FSHR requires site-specific phosphorylation of the cytoplasmic *C*-terminal domain of the receptor at serine and threonine residues by various kinases of the GRK-family. Moreover, depending on the set of phosphorylation sites in the *C*-terminal domain, β-arrestins can cause either desensitization of FSHR or its inclusion in signaling complexes in early endosomes [201–203].

In the nonphosphorylated state, FSHR is unable to bind effectively to β-arrestins. This distinguishes it from LHCGR, which is able to recruit β-arrestins while in a non-phosphorylated state, making LH-regulated β-arrestin pathways independent of the pattern and activity of GRKs [204]. The interaction of LHCGR with β-arrestins involves the receptor ICL3 and ADP-ribosylating factor 6 (ARF6) [22,204–206]. In the inactive, GDP-bound state, ARF6 binds to β-arrestin, and the resulting complex is anchored in the plasma membrane, being available for interaction with cytoplasmic regions of LHCGR. After activation of LHCGR by gonadotropin, a transient complex is formed between the receptor and the ARF6 protein, which leads to the exchange of GDP for GTP at the nucleotide-binding site of ARF6. In this case, a reorganization of the complex occurs between the active, GTP-bound form of ARF6 and β-arrestin, as a result of which the latter is released from the complex with ARF6 and binds to ICL3 of LHCGR, which ensures down-regulation and internalization of the ligand-receptor complex [203,206].

A comparative analysis of the effects of LH and hCG on the recruitment of β-arrestins demonstrates that hCG is more active in this regard, causing β-arrestin-mediated effects much faster and at lower concentrations (Table 4). This is indicated by the EC_{50} value for hCG-induced βarr2 recruitment in mouse Leydig tumor cells (about 10 nM), which is 13 times lower than that for LH (130 nM). Moreover, based on the magnitude of the stimulatory effect on β-arrestin recruitment, LH can be classified as a partial agonist of LHCGR, in contrast to hCG, which exhibits agonistic activity for β-arrestin-specific signaling [13]. As noted above, cAMP-dependent pathways play a key role in stimulating steroidogenesis, and hCG is a more effective activator of steroidogenesis compared to LH up to the stage including progesterone synthesis. In this regard, it is of interest that β-arrestins positively regulate the synthesis of progesterone by gonadotropins, since the reduction of their expression by microRNAs leads to a significant inhibition of the production of this steroid hormone, a precursor of androgens and estrogens [13]. hCG, in comparison with LH, more effectively stimulates the recruitment of β-arrestins, as a result of which this effect, along with more pronounced

Table 4. Comparison of the effects of luteinizing hormone (LH) and human chorionic gonadotropin (hCG) on β-arrestin pathways – similarities and differences.

Similarities
Following activation of LHCGR, both gonadotropins, LH and hCG, induce the recruitment of β-arrestins, thereby internalizing and down-regulating the activated receptor. Along with this, activation of LHCGR by gonadotropins can induce intracellular (endosomal) signaling, which involves β-arrestins.
Differences
(1) The stimulating effects of hCG on the recruitment of β-arrestins are more pronounced in comparison with those of LH and are realized at lower concentrations, and therefore hCG is considered as a full agonist in relation to the recruitment of β-arrestins, while LH is a partial agonist. (2) hCG strongly stimulates β-arrestin-mediated internalization and down-regulation of LHCGR, while the corresponding effects of LH are much less pronounced, including even with prolonged exposure to LH at relatively high concentrations. The result of this is multiple aggregation of receptors in endosomes, which leads to a weakening of cAMP signaling during long-term hCG-induced activation of LHCGR.

Note: cAMP, cyclic adenosine monophosphate; LHCGR, luteinizing hormone/chorionic gonadotropin receptor.

stimulation of AC, can provide higher steroidogenic activity of hCG, which, however, is limited to the stage of progesterone synthesis. At later stages of testicular and ovarian steroidogenesis, the steroidogenic activity of hCG becomes comparable to that of LH, resulting in similar final steroidogenic responses to LH and hCG drugs observed in the clinic [163].

The lower activity of LH in relation to β-arrestins makes it possible to prevent or at least reduce the internalization and down-regulation of LHCGR during prolonged exposure to even relatively high concentrations of LH, thereby maintaining the sensitivity of target cells to endogenous gonadotropins, and preventing depletion of the pool of functionally active LHCGRs both in the plasma membrane and in intracellular compartments (Table 4). Activating mutations in LHCGR, including the replacement of the Asp^{564} located in ICL3 with glycine or tyrosine, cause a significant (in the case of $Asp^{564}Gly$ fivefold) increase in the degree of internalization of constitutively activated LHCGR into the cell [207]. It is important to note that activating mutations induce the same conformations of LHCGR as hCG, as indicated by the lack of significant effect of hCG on the redistribution of mutant LHCGR in membrane microdomains, which is due to their interaction with β-arrestins [208].

The localization of the β-arrestin-competent region in ICL3 of LHCGR and the key role of the Asp^{564} residue in β-arrestin binding are also indicated by the results of studying synthetic peptides corresponding to various regions of ICL3 [204,209]. Using surface plasmon resonance, it was shown that the peptide corresponding to the central part of ICL3 binds specifically to βarr2 in picomolar concentrations, while its binding to βarr3 occurs with significantly lower affinity, in millimolar concentrations. Substitutions of the Asp^{564} with glycine, asparagine, glutamic acid, and leucine suppressed the specific binding of the "mutant" peptide to βarr2, indicating its functional importance for the formation of the βarr2-binding surface of LHCGR [204].

6. *N*- and *O*-Glycosylation of Gonadotropins, Their Role in Signal Transduction and Possible Allosteric Effects

6.1 Common Features of Glycosylation of Gonadotropin Subunits

Of decisive importance for the functional activity of gonadotropins and their signaling cascades is the process of *N*-glycosylation at asparagine residues, and in the case of hCG and other hCGs, also the process of *O*-glycosylation at serine-containing sites localized in the *C*-terminal segment of their β-subunits. As noted above, α-GPH, common to LH and hCG, undergoes *N*-glycosylation at two sites, where the targets of glycosyltransferases are residues Asn^{52} and Asn^{78}, while β-LH and β-hCG have one and two sites for *N*-glycosylation (Fig. 2). Along with differences in the degree of glycosylation and localization of glycans in the α- and β-subunits, the nature and charge of oligosaccharide chains play an equally important role in gonadotropin-induced signal transduction. For example, in the α- and β-subunits of LH and the sulphated hCG, a significant part of the oligosaccharide chains contain terminal residues of sulphated GalNAc, which have a significant negative charge, while at the ends of *N*-glycans in classical hCG and in the α- and β-subunits of FSH, mainly sialic acid residues are localized, the negative charge of which is not so pronounced in comparison with sulphated GalNAc [76]. The total charge of the *N*-glycan is determined by the number and ratio of terminal glycosyl residues, which, in turn, depends on the degree of branching of the *N*-glycan. The *N*-glycans in the α- and β-subunits of LH and hCG are significantly less branched than those in the FSH, and this not only makes a significant contribution to the resulting charge of these subunits, but also determines their spatial configuration and ability to bind to the orthosteric site LHCGR.

It should, however, be taken into account that the structure of *N*-glycans in the α- and β-subunits that form dimeric gonadotropin can vary greatly, which is due to differences in their post-translational processing, includ-

ing in the machinery of their *N*-glycosylation, as well as in the possibility of exchange and reassociation of these subunits. Thus, recent studies of the pattern of *N*-glycans in FSH using mass spectrometry have shown that β-FSH have fucose-modified *N*-glycans, of which approximately one third are weakly branched bi-antennary glycans, and another third are highly branched tri-antennary glycans and tetra-antennary glycans [23]. In turn, *N*-glycans in α-FSH do not contain fucose residues and are bi- and triantennary, and the Asn^{56} residue in the subunits may have a massive *N*-glycan corresponding in mass to tetra-antennary structures, but in reality is tri-antennary, additionally containing many lactosamine repeats [23]. The glycosylation pattern and chemical structure of *N*-glycans influence the folding of gonadotropins, their maturation and intracellular transport, the stability and lifetime of gonadotropins in the bloodstream, and are also the most important regulatory mechanisms that control their binding to the receptor and specific biological activity [18–20,22,23,210–214].

Taking into account the high variability of glycosylation of the α- and β-subunits of gonadotropins [19,23,86,211,213,215–217], the total number of glycoforms of $\alpha\beta$-heterodimer complexes of LH and hCG can be very significant even in one organism and amount to tens, hundreds and even thousands of variants [19,218]. The interaction of gonadotropins with LHCGR is highly dependent on their glycosylation, as a result of which the resulting effect of a gonadotropin is directly dependent on the ratio and pattern of its glycoforms. Taking into account the peculiarities of the location of *N*-glycans in gonadotropins, as well as *O*-glycans in the *C*-terminal part of hCG and equine gonadotropins, there is every reason to believe that glycosyl components are able to interact with receptor sites other than its orthosteric site, thereby exerting an allosteric effect on the affinity of gonadotropin for LHCGR, on the bias of intracellular signaling and the efficiency of the cellular response [18,20,22,23,219]. This is of great importance for assessing the specific biological activity of recombinant human LH and hCG, as well as hCG isolated from urine when used in ARTs [19]. In addition, the hCG glycosylation pattern is important for monitoring the course of the first trimester of pregnancy, assessing possible risks for embryo development and pregnancy complications [74,220–227]. The need to match recombinant forms of LH and hCG [19], as well as chemically synthesized hormones with LH activity [228], with natural glycoforms is one of the most difficult problems of modern gonadotropin-based pharmacology, being the main obstacle to create physiologically relevant LH and hCG drugs.

Most of the data on the effect of *N*-glycosylation on the binding characteristics and activity of gonadotropins were obtained in relation to FSH, which is largely predetermined by the intensive development and characterization of pharmacological preparations of FSH, primarily recombinant FSH, for use in ARTs [23]. And these data indicate that *N*-glycans may play the role of the most important allosteric modulators of FSH signaling, controlling its efficiency and bias, and this is based both on the study of FSH isoforms that differ in the degree of *N*-glycosylation and the structure of *N*-glycans, and on the study interactions of highly and weakly glycosylated FSH dimers with the FSHR ectodomain [23,229,230].

Despite some differences in the structural organization of FSH and its receptor, as well as *N*-glycans that modify α- and β-FSH, from those of LH, hCG and LHCGR, the main patterns of the influence of *N*-glycosylation on functional activity are also valid for gonadotropins with LH activity. Normally or weakly glycosylated forms of hCG stimulate cAMP-dependent signaling pathways with greater efficiency and, as a result, they are effective stimulators of the synthesis of steroid hormones, while hyperglycosylated forms of hCG are less active in this regard [21], and their regulatory effects are realized largely in relation to the stimulation of mitogenic pathways, which may involve receptors other than LHCGR [231]. In this case, the stability of various forms of hCG also plays an important role, since normally or weakly glycosylated forms of the hormone have a significantly shorter half-life in the bloodstream, in contrast to hyperglycosylated forms, in which sites for proteolytic cleavage are protected by many glycosyl groups [74,232]. Moreover, important information is provided by studying the interaction of glycosylated forms of TSH with TSHR, which is structurally similar to LHCGR [233]. It is quite interesting that hyperglycosylated forms of hCG, but not normally glycosylated forms, are able to stimulate TSHR, increasing the level of thyroid hormones, which may cause an increased risk of miscarriages and the development of autoimmune thyroid diseases [234], especially in the case of expression of the TSHR variant with the replacement of Val^{597} with an isoleucine in the TMD, leading to hypersensitivity to hCG [235].

The following facts and theoretical principles support the allosteric nature of the influence of *N*- and *O*-glycosylation of gonadotropins on their activity and regulatory properties.

6.2 Effect of Gonadotropin Glycosylation on Its Binding to the Receptor

A modulating role of *N*-glycans is postulated in the process of hormone binding to the receptor, without direct interaction with the orthosteric site. It is assumed that high-affinity binding of a glycoprotein hormone (GPH) to the orthosteric site of a glycoprotein hormone receptor (GPHR) should involve almost exclusively specific interactions between their specific amino acid residues or their clusters, that is, based on the principles of protein-protein interaction [236–238]. The interactions between the *N*-glycan and the polypeptide chain in this case cannot be highly specific and high-affinity, since the structure of *N*-glycans is very variable, and therefore they can be involved in the interaction of

the hormone with the orthosteric site only indirectly, modulating its binding through allosteric mechanisms. Indeed, deglycosylated forms of gonadotropins retain the ability to bind to the receptor, and in some cases their affinity even exceeds that of glycosylated forms [239,240].

Extracellular regions of the receptor can also be targets for oligosaccharyltransferases, which suggests the possibility of glycan-glycan interactions between glycosylated forms of the hormone and glycosylated sites of the receptor ectodomain. Taking into account the dominance of the negative charge in *N*-glycans that modify gonadotropins and their receptors, the glycosyl components of the hormone and the receptor will be subject to mutual repulsion, as a result of which their role in stabilizing the ligand-receptor complex seems unlikely, being limited, most likely, to the negative control of the formation such a complex. In agreement with this are the results obtained using molecular modeling and structural-phylogenetic analysis for the GPHs and GPHRs, including for TSH and its receptor, about the absence of *N*-glycans in the hormone binding sites of GPHRs. Thus, in all studied vertebrate species in all types of GPHRs, including LHCGR, there are four regions free of *N*-glycans, including two on the concave faces of the LRR subdomain, responsible for high-affinity binding of the hormone [238]. At the same time, *N*-glycans can influence the relative position and efficiency of interaction between the binding sites of the hormone and the receptor, including by changing the location of the receptor ectodomain in relation to its TMD. All this will influence the pattern of active conformations of the ligand-receptor complex, thereby determining the activation of intracellular cascades and the predominance of signal transduction [23,241].

6.3 The Influence of Gonadotropin Glycosylation on the Stability of Active Conformations of the LHCHR and on the Conadotropin-Regulated Intracellular Cascades

The *N*-glycosylation directly affects the effectiveness of the regulatory influence of gonadotropins on intracellular effector systems, including cAMP-dependent signaling cascades and calcium signaling, and, as a consequence, the magnitude and nature of the physiological response. Moreover, the presence or absence of *N*- and (or) *O*-glycans can change the pharmacological profile of gonadotropin, which was first demonstrated more than thirty years ago. It was shown that deglycosylation of gonadotropins leads to a decrease or complete loss of their specific activity [242], and in some cases, deglycosylated forms of gonadotropins even acquired the properties of LHCGR antagonists [210,243]. It should be emphasized that the binding of deglycosylated gonadotropins to receptors was preserved in most cases, although its affinity, as a rule, changed significantly, and this again indicates the allosteric nature of the influence of glycans on the activity of GPHRs.

One of the key molecular mechanisms that may mediate the influence of the degree and pattern of *N*-glycosylation of gonadotropins on their ability to activate the receptor is the influence of *N*-glycans on the relative position of the ectodomain and TMD of the receptor. *N*-glycosylation of gonadotropin may stabilize the active conformation of gonadotropin-bound receptor, allowing efficient interaction between the hinge region of the ectodomain and the ECL1 and the outer vestibule segments of the TMD. In this case, *N*-glycosylation contributes to the activation of the signaling cascade, and the gonadotropin functions as a full or partial agonist. Otherwise, when a gonadotropin has an *N*-glycosylation pattern that prevents this interaction, it functions as an antagonist or inverse agonist.

Deciphering the possible mechanisms of the influence of *N*-glycans on the pharmacological profile of gonadotropins showed that the *N*-glycan, which modifies the Asn^{56} residue in α-GPH, can play a decisive role here. This is supported by the fact that a similar mechanism is implemented for different pairs of GPHs and GPHRs, which have identical α-subunits in their primary structure and different β-subunits. In 2022, the role of the *N*-glycan at position 56 of the TSH α-subunit in TSHR was established [244]. It has been shown that TSH, which includes the Asn^{56} in α-TSH modified by a large *N*-glycan (up to 16 monosaccharide units), when interacting with TSHR, stabilizes its active conformation by moving the LRR subdomain from the outer vestibule to the TMD, facilitating the interaction of the latter with hinge region. The reason for this is the strong steric and possibly electrostatic repulsion between the bulky *N*-glycan of α-TSH, directed orthogonally to the membrane surface, and membrane phospholipids. The absence of this *N*-glycan does not allow stabilization of the active conformation or leads to a minor effect on the equilibrium of active and inactive conformations, which prevents TSHR activation and TSH-induced stimulation of AC and other effector proteins [244]. To prove the exceptional importance of the glycosyl component, experiments were carried out with the Fab fragment of antibodies K1-70 with the activity of weak TSHR agonists [244], which, when combined with TSH and stimulating antibodies, for example, with K1 -18, exhibited antagonistic properties [245,246]. *N*-Glycosylation of antibodies K1-70, which in terms of exposure of *N*-glycan and its structural organization had similarities with glycosylation of the Asn^{56} in α-GPH (α-TSH), led to a sharp increase in the ability of such modified antibodies to stimulate the AC signaling system, due to whereby they acted as full agonists. Thus, the hypothesis about the role of *N*-glycan at position 56 of α-GPH in stabilizing the active form of receptors of the GPHR family, and possibly other receptors including ectodomains with multiple LRRs, was confirmed [244].

In relation to FSHR, the role of interactions of α-GPH *N*-glycans (α-FSH) with the membrane phase is also considered as one of the mechanisms for stabilizing the active state of FSHR, and here the structure and charge of the *N*-glycan modifying Asn^{56} in α-GPH (α-FSH) is important

[23]. It was established that in this position in various glycoforms of FSH a bulky three-antennary *N*-glycan is localized, which is critical for the biological activity of the hormone, which, as in TSHR, is orthogonal to the surface of the plasma membrane. Interestingly, both *N*-glycans localized in β-FSH, as well as Asn^{56} of α-FSH, are located in the part of the FSH dimer that faces the membrane. They are at an angle to the surface of the plasma membrane, and not orthogonal to it, and therefore are only partially localized in the cavity formed by the ectodomain and the outer vestibule of the TMD. In accordance with this, they do not create significant steric tension in the ligand-receptor complex. Despite this, deglycosylation of β-FSH at *N*-glycosylation sites has a significant impact on FSH activity, including through changes in the localization and rigidity of the Asn^{56}-coupled glycan and subtle reorganization of the entire ligand-receptor complex. This is illustrated by the studies on the preservation or even significant increase in biological activity of FSH dimers deglycosylated at one or two sites of β-FSH, and it has been shown that the increase in dimer activity is positively correlated with an increase in the affinity of hypoglycosylated forms of FSH for the receptor [239,247–249].

It has been established that hypoglycosylated forms of FSH with a β-subunit having a mass of 18 or 21 kDa and, accordingly, deglycosylated at positions Asn^{7} and Asn^{24}, stimulate FSHR and the cascades dependent on it with significantly higher activity in comparison with the highly glycosylated form of FSH with a mass of 24 kDa, which directly affects their physiological effects, as well as the pharmacological profile in ARTs [23,240,249–253]. It can be assumed that the loss of one of the *N*-glycans in β-FSH increases the flexibility of the molecule and ensures its more efficient anchorage in the cavity between the extracellular and transmembrane domains with subsequent activation of FSHR. In turn, the *N*-glycan at position 78 of α-FSH, facing away from the membrane and located outside the cavity formed by the ectodomain and TMD, does not affect the activity of the FSH dimer [247,248].

With regard to LHCGR, it has also been established that when it binds to hCG, the ectodomain rotates by a mechanism similar to that of FSHR and TSHR. Cryogenic electron microscopy data for LHCGR and TSHR have demonstrated similar rotation angles of the main core of the ectodomain during the transition from the active to the inactive conformation, at 45° and 38°, respectively [28,244]. The similarity in the size of *N*-glycans at position Asn^{56} of α-GPHs in gonadotropins and TSH shows that they perform a similar function in the process of receptor activation. Numerous studies on the effect of deglycosylation of α-GPHs in the hCG and LH dimers on LHCGR activity, as well as the results of studies of mutant and hybrid forms of hCG and LH largely confirm this mechanism of participation of glycans of the α-subunit in the binding and activation of LHCGR.

The functional importance of the Asn^{56}, the target of *N*-glycosylation, in α-GPH included in the hCG dimer is supported by data on the activity of hCG lacking this site [254,255]. More than thirty years ago, Irwing Boime's group [254,255] found that this site for *N*-glycosylation plays an extremely important role both in the formation of the heterodimeric hCG complex and for the activation of cAMP-dependent pathways and the regulation of steroidogenesis. Heterodimers containing a normally glycosylated β-subunit and an α-subunit lacking an *N*-glycan at position Asn^{56} were characterized by a significantly reduced ability to increase intracellular cAMP levels and activate steroidogenesis. In the same case, when the β-subunit was deglycosylated, hCG not only completely lost the ability to stimulate AC and induce steroidogenesis, but also acquired the properties of a competitive antagonist, interfering with the regulatory effects of normally glycosylated hCG [255]. The authors noted that the absence of a glycan at position Asn^{78} of the α-subunit, as well as in both sites for *N*-glycosylation in the β-subunit (Asn^{13}, Asn^{30}) was less critical for the steroidogenic activity of hCG, but only if the site Asn^{56} remained glycosylated (Fig. 2). It was also found that of the two sites for *N*-glycosylation present in the β-subunit, the Asn^{13} site is more important for activation of the AC signaling system [255].

A significant decrease in LH-like activity upon deglycosylation of Asn^{56} was also shown for the $\alpha\beta$-heterodimer of equine CG, which contained a normally glycosylated β-subunit (eCGβ/$\alpha\Delta$56), both when assessing the ability of the mutant dimer to stimulate testosterone production in a primary culture of Leydig cells, and when assessing its ability to stimulate aromatase in cultured ovarian granulosa cells [90,256]. At the same time, the mutant dimer also had reduced FSH-like activity, characteristic of eCG [90]. Treatment of gonadotropins with peptide-*N*-glycanase F, which cleaves off *N*-glycan, including at position 56 of α-CG, also led to the loss of gonadotropin biological activity [90]. Deletion of the *C*-terminal region of equine β-CG, which contains numerous sites for *O*-glycosylation (eCGβ-D/α), led to impaired secretion of the mutant gonadotropin, but had a lesser effect on the functional activity, while the double mutant (eCGβ-D/$\alpha\Delta$56), which lacked the *O*-glycosylation site in β-CG and the Asn^{56} site in α-CG, was completely devoid of both LH- and FSH-like activities [90]. The EC_{50} values for the eCGβ/$\alpha\Delta$56 and eCGβ-D/$\alpha\Delta$56 mutants were reduced by 2.1 and 3.4 times, and the number of binding sites on the cell surface was 56 and 65% of those for wild-type CG [257].

It was noted above that equine LH, like equine CG, has both LH- and FSH-like activity. At the same time, the same changes in the glycosylation status of equine LH can have different effects on these LH- and FSH-like activities, as has been demonstrated using the example of hybrid gonadotropin molecules. Hybrids that included equine α-LH and α-FSH deglycosylated at Asn^{56}

and *C*-terminally truncated equine β-LH and β-hCG that were unable to undergo *O*-glycosylation, as well as hybrids that were different combinations of mutant subunits with wild-type subunits were studied [92]. Hybrids N(56)dg-eLHalpha:eLHbeta(t) and N(56)dg-eFSHalpha:eLHbeta(t) bound to LHCGR with an affinity similar to that of wild-type LH, but had a very low ability to stimulate testosterone synthesis in Leydig cells and stimulate progesterone production and aromatase activity in ovarian granulosa cells. The hybrid N(56)dg-eLHalpha:eLHbeta(t) demonstrated an inhibitory effect on the stimulation of ovarian steroidogenesis caused by FSH, and was significantly superior in this regard to N(56)dg-eLHalpha:eLHbeta and N(56)dg-eCGalpha:eLHbeta(t). Interestingly, N(56)dg-eLHalpha:eCGbeta did not have an inhibitory effect on the effects of FSH and, at relatively high concentrations, retained the ability to increase the expression and activity of aromatase [92]. It is possible that in the latter case, a certain role compensating for the absence of *N*-glycan at the Asn^{56} residue of α-eLH is played by the tail of equine β-CG, glycosylated at serine residues. Thus, the glycosyl components in hCG dimers can, to a certain extent, compensate or modulate each other's functions.

6.4 The Influence of Glycosylation on the Heterodimerization, Receptor Specificity and Stability of Gonadotropins

The important role of *N*-glycans in the formation and stability of heterodimeric gonadotropin complexes has been demonstrated. This determines the affinity of gonadotropins for the receptor, affects the bias of signal transduction, being one of the allosteric mechanisms of gonadotropin signaling, and is also essential for the bioavailability and pharmacokinetic characteristics of gonadotropins. Long-term incubation (72 hours) of cell cultures with the N(56)dg-eLHalpha:eFSHbeta hybrid, in which equine α-LH was deglycosylated at the Asn^{56} residue, led to an unstable dimeric complex with β-FSH and a complete loss of activity of the hybrid gonadotropin [92].

At the same time, for hCG, the β-subunit of which was *O*-glycosylated, *N*-glycosylation of α-hCG at the Asn^{56} residue did not significantly affect the stability of the hCG heterodimer, although it was critical for its activity. The dissociation constants for β-hCG complexes with normal and Asn^{56}-deglycosylated α-hCG were similar, and CD spectroscopy and NMR data showed only slight differences for these complexes, mainly associated with local changes in the spatial organization of the AAR cluster of β-hCG, potentially contacting the *N*-glycan at the Asn^{56} site [258]. At the same time, the biological activity of the hCG heterodimer with Asn^{56}-deglycosylated α-hCG was sharply reduced, which confirms the importance of the *N*-glycan at this position for interaction with LHCGR, in accordance with the model proposed for FSHR and TSHR [23,244]. Crosslinking of β-CG with a mutant α-subunit with the replacement of the Thr^{54} with alanine, which prevents normal glycosylation at Asn^{56}, resulted in a single-chain phCGalphabeta molecule. This construct was characterized by normal binding of phCG$\alpha\beta$ to LHCGR and was active, while the dimer with such a mutation was slightly active [259]. Thus, the covalent crosslink of mutant subunits with reduced resistance to the formation of active dimeric complexes can ensure their preservation of functional activity. It should also be noted that during the formation of such a single-chain gonadotropin, a number of other mutations critical for the activity of hCG did not cause a significant decrease in its specific activity. This suggests that changes in the localization and accessibility of molecular determinants, including those represented by the glycosyl moiety, mediating allosteric interactions of gonadotropin with the receptor, can critically affect the affinity and effectiveness of hCG.

Hyperglycosylation makes an even more significant contribution than deglycosylation to the ability of hCG to form heterodimeric complexes and plays a critical role in the pattern of their specific biological activity [260], especially given the very large set of glycans involved in the modification of the hCG molecule [213]. In the case of the α-hCG subunit, it has been shown that excessive glycosylation leads to disruption of its association with β-hCG, which was first shown back in 1990s [261]. Monomeric α-hCG, containing various bulky *N*-glycans that prevent complex formation, is secreted by the placenta, pituitary gland, and some tumors [260,262], and have also been identified in significant quantities in seminal fluid [263]. It has been shown that *N*-glycans in hyperglycosylated hCG subunits, which are enriched in seminal fluid, contain a large number of fucose residues, which significantly changes the conformational mobility of both the glycans themselves and the hCG subunits [264].

Hyperglycosylated α-GPH (α-CG) can function in the form of both monomers and homodimers of α-CG–α-CG, which is also true for hyperglycosylated β-CG. It has been shown that hyperglycosylation of β-CG not only disrupts the stability of their complex with the α-subunit, but also promotes the generation of free forms of β-CG and their homodimers β-CG–β-CG. At the same time, hyperglycosylated hCG, including a population of monomeric β-hCG, not only interacts with LHCGR with lower affinity [21], but also acquires the ability to effectively stimulate transforming growth factor-β (TGFβ) receptors [75,232,265]. Stimulation of these receptors, along with LHCGR-mediated stimulation of cAMP-dependent pathways and various isoforms of PKC, leads to the activation of post-receptor effector proteins such as ERK1/2, Akt kinase and SMAD (Similar to Drosophila Mothers Against Decapentaplegic) family proteins [266]. This determines the powerful mitogenic and anti-apoptotic potential of hyperglycosylated forms of β-CG, which is realized independently of signaling pathways including LHCGR and makes a decisive contribution to the

growth and differentiation of the embryo in the early stages of its development. By acting on TGFβ receptors, hyperglycosylated hCG functions to a large extent as an auto- and paracrine factor [231,232,265]. In an unfavorable scenario, hyperglycosylated forms of β-CG may be involved in tumorigenesis and provoke the development of gonadotropin-dependent tumors [267–269].

The different receptor specificities of the weakly and highly glycosylated hCG isoforms are believed to be due to differences in the accessibility of certain regions of the molecule to interact with LHCGR, which allows the hyperglycosylated form of hCG to activate the TGFβ receptor. Thus, the B-152 antibody, which specifically recognizes *O*-glycans bound to residue 132 in the *C*-terminal part of β-hCG, is unable to precipitate with hCG heterodimers [270,271]. This indicates the release of the *C*-terminal region in hyperglycosylated forms of hCG, either due to the dissociation of β-hCG from the dimeric complex, or due to a significant change in the conformation of the latter, ensuring the presentation of this region for interaction with antibodies. Along with this, hyperglycosylation leads to a decrease in the affinity of hCG for LHCGR and a weakening of its stimulating effects on the effector systems of the target cell, which can be considered as an allosteric effect of *N*- and *O*-glycans on receptor binding and the specific activity of the hormone. The EC_{50} for LHCGR activation in the case of classical hCG with normal glycosylation status was about 3.3 pM, while for hyperglycosylated forms of the hormone it was from 7.1 to 14.0 pM [21]. Thus, the excess amount of glycans in the hCG functions as a tethered NAM in relation to the activation of LHCGR.

It is important that under the *in vivo* conditions, a significant contribution to the activity of hCG can be made by the influence of the *N*- and *O*-glycosylation pattern on post-translational processing, secretion, stability and bioavailability of gonadotropin. As noted above, in equine β-CG, the *C*-terminal *O*-glycosylation sites are critical for normal gonadotropin processing and secretion, as demonstrated by studies of the eCGβ-D/α dimer mutant [90,256,257]. Based on this, chimeras were constructed in which the *C*-termini of the α- and β-subunits forming heterodimers of eel LH or FSH were linked to each other using the *C*-terminal sequence of equine β-CG, containing functionally active sites for *O*-glycosylation [272]. This led to a mutant gonadotropin (LH-M), which, on the one hand, was able to be secreted efficiently and quickly, and on the other, had the ability to bind to the LHCGR and stimulate the AC signaling pathway, increasing the intracellular level of cAMP. Moreover, the AC stimulating effect and EC_{50} value for LH-M were not inferior to those obtained for wild-type LH [272]. However, further studies are needed to assess the effectiveness of such chimeric gonadotropins, including taking into account their effect on other LH-dependent signaling cascades.

6.5 The Influence of Glycosylation on the Receptor Complex Formation

It should be noted that *N*-glycosylation and other modifications of gonadotropin receptors can also affect the stability of homo- and heterodi(oligo)meric receptor complexes. Despite the fact that gonadotropin receptors can function as monomers, their formation of homo- and heterodi(oligo)meric receptor complexes largely determines their functional activity, and the ratio and structural organization of such complexes plays an important role in the pattern of signal transduction, especially in the case of heterodimers, formed by LHCGR and FSHR (see Section 7). Various types of glycan-mediated interactions may be involved in the stabilization of receptor complexes, including glycan-protein and glycan-glycan interactions, which can stabilize or, conversely, destabilize the association of protomers within the receptor complex or in higher-order complexes.

For the FSH–FSHR system, it has been shown that the process of formation of receptor complexes may also depend on the degree of glycosylation of the hormone. The FSHR binding to high concentrations (30 nM) of hypoglycosylated forms of FSH containing 18 and 21 kDa β-FSH leads to an increase in the proportion of monomeric forms of FSHR, from 70% in the ligand-free state to 80% in FSH-bound state, and this effect is realized after only 2 min and positively correlates with the FSH-induced activation of cAMP production [214]. Highly glycosylated FSH, including 24 kDa β-FSH, is less effective in this regard, and an increase in the proportion of the monomeric form of FSHR and activation of cAMP signaling are achieved much later. The deglycosylated form of equine LH, including eLHα deglycosylated at Asn^{56} and eLHβ lacking the *C*-terminal segment 121–149 containing sites for *O*-glycosylation, on the contrary, increases the proportion of the oligomeric form of the receptor to 50% and higher. Exposure of FSHR to low concentrations (1 nM) of weakly glycosylated forms of FSH leads to the same result [214]. All this indicates that the degree of FSHR oligomerization depends not only on the degree of *N*-glycosylation of gonadotropin, but also on its concentration, and demonstrates a relationship between *N*-glycosylation and the kinetic parameters of FSHR complex formation. All of the above may also be true for LHCGR, especially in terms of its heterodimerization with FSHR, and this requires further research.

6.6 Impact of Gonadotropin Glycosylation on Signal Transduction Bias

The degree and pattern of *N*-glycosylation of gonadotropins can have a significant impact on signal transduction bias, as well as determine the fate of the ligand-receptor complex in the cell, which largely depends on the nature of the interaction of ligand-activated LHCGR and other GPHRs with β-arrestins. Most of the evidence on

the influence of *N*-glycosylation on intracellular signaling bias and cellular response selectivity, and more broadly on folliculogenesis and oogenesis, relates to FSH and its signaling cascades [214,230,253,273,274]. At the same time, there is, although indirect, data regarding gonadotropins with LH activity. Thus, in the middle of the menstrual cycle in women, at the stage of ovulation induction, there is not only a significant increase in the total concentration of LH glycoforms, but also a predominance of the proportion of the weakly glycosylated form of the hormone, containing two *N*-glycans in the α-subunit (on average 62–65%). This is synchronized with a powerful activation of LH-dependent signaling pathways in ovarian cells, leading to follicle rupture [20]. At the same time, at the early follicular and luteal phases, both the total concentration of LH in the blood and the proportion of its weakly glycosylated form (LHdi) sharply decrease, which coincides with a weakening of LH-dependent signaling in the ovaries. It is important to note that by the 14th day of the cycle, the ratio of terminal sialic acids and sulphated GalNAc in *N*-glycans, which determine the total negative charge of LH molecules, significantly changes, and this also makes a significant contribution to their interaction with LHCGR [20].

When studying equine LH with LH/FSH-like activity, evidence was obtained of signaling bias of its glycosylated and Asn^{56}-deglycosylated forms in relation to the cAMP-dependent and β-arrestin pathways, which are mediated through activation of FSHR [275]. At the same time, deglycosylation led to a significant decrease, compared with the native hormone, in the stimulating effect of equine LH on the AC activity and downstream cAMP-dependent cascades, and at the same time maintained and even potentiated the activation of the β-arrestin pathway, resulting in activation of ERK1/2 and increased phosphorylation of the ribosomal protein rpS6. Phosphorylation of rpS6 was carried out independently of PKA through a signaling pathway including mTOR-mediated phosphorylation and activation of ribosomal kinase p70S6K [275].

6.7 Some Comments on the Role of Gonadotropin Glycosylation in LHCGR Signal Transduction

Thus, glycosylation has a significant impact on the stability of heterodimeric gonadotropin complexes, on their binding characteristics and on the interaction with LHCGR. Moreover, in the case of hyperglycosylated forms of β-CG, the receptor specificity of gonadotropins changes, with an increase in the degree of glycosylation, shifting from LHCGR to TGFβ receptors. It is also possible that *N*-glycosylation of extracellular regions of LHCGR influences the formation and activity of the ligand-receptor complex. However, it is not always possible to separate the allosteric effects of glycosylation from steric effects affecting the accessibility of interaction sites of gonadotropin and its receptor, and from the influence of glycosylation on post-translational processing and secretory activity of gonadotropins, as well as on the processing and translocation of the receptor into the plasma membrane. In addition, the effect of glycosyl residues largely depends not only on their quantity and location, but also on the type of glycosylation and the chemical structure of glycans (the ratio of sialic acid and sulphated GalNAc, which determines the charge of *N*-glycans, and the presence of fucose residues, which determines them conformational rigidity, etc.). Attempts have been made to separate the functions of *N*- and *O*-glycosylation for the activity and structural organization of gonadotropins [210], but still, due to objective circumstances, this remains a very difficult task. This is due both to the fact that the results obtained in the *in vitro* cannot always be relevantly translated into the bioactivity of gonadotropins and their signaling systems in the *in vivo*, and to the fact that the structural diversity of *N*- and *O*-glycans and the multiplicity of enzymatic systems responsible for the modification and processing of their oligosaccharide backbone make a significant, and in certain cases decisive, contribution to the stability and pattern of specific activity of gonadotropins.

It is also important that glycosylation, as noted above, critically affects the pharmacokinetics of gonadotropins and their resistance to proteolytic degradation, which also affects the effectiveness of their regulatory effect on intracellular signaling. In addition, during complicated pregnancy and in conditions of reproductive dysfunction, as well as when taking various drugs, including contraceptives, significant changes in the glycosylation pattern are observed, which inevitably affects the pharmacokinetics, bioavailability and pattern of gonadotropins with LH activity, primarily various forms of hCG, and mediates the various effects of LH and hCG glycoforms on steroidogenesis, folliculogenesis, oogenesis and embryogenesis [276–279].

7. Formation of Receptor Complexes as an Allosteric Mechanism for LHCGR Regulation

7.1 Common Principles of the Formation of Homo- and Heterodimeric Receptor Complexes

The formation of homo- and heterodi(oligo)meric GPCR complexes, as well as their complexes with other signal proteins (G proteins, β-arrestins, RAMPs, etc.) plays an important role in signal transduction, and a significant contribution is made here by allosteric influences [42,280–283]. Thus, there is numerous data on the effect of complex formation between class C GPCRs on their functional activity and binding characteristics [282,284–286]. Complex formation has been less studied for class A GPCRs, which include LHCGR and receptors for other pituitary glycoprotein hormones. The prevailing view here is that class A GPCRs are active predominantly in monomeric form, while their formation of complexes is due to their transition to an inactive state and/or is necessary to modulate the binding

characteristics and effectiveness of the orthosteric agonist [42,280,281,283], as demonstrated for rhodopsin, one of the most structurally simple representatives of class A GPCRs [287,288]. Homodi(oligo)merization may also be involved in the desensitization, processing, and translocation of class A GPCRs.

At the same time, there are good reasons to believe that, depending on the type of class A GPCRs, their formation of homo- and heterodi(oligo)meric complexes may play a more significant role in signal transduction than signal modulation alone. It may provide a mechanism for trans-activation of receptors, make a significant contribution to biased activation of intracellular cascades and, especially in the case of heterocomplex formation, mediate multidirectional regulation of these cascades [289,290]. Moreover, the molecular mechanisms of the influence of protomers on the functional activity of complexes also include allosteric interactions [280,283,291]. The formation of receptor complexes itself is under the control of various allosteric regulators, specifically interacting with sites in protomers that form contacts between them, including sites interacting with membrane lipids and adapter proteins [288,292].

Homodimeric complexes have been characterized for many members of class A GPCRs, including rhodopsin [287,288] and histamine H3 receptors [293]. Homo- and heterodimeric complexes form different subtypes of β-adrenergic receptors, and this is of great importance for the adrenergic regulation of the functions of the cardiovascular and other systems [294,295]. Using various approaches, heterocomplexes have been identified between the D2-dopamine receptor and the protomers of A2A-adenosine [296,297], CB1-cannabinoid [298] and oxytocin receptors [299], between the 5-HT2A-serotonin receptor and the protomers of 5-HT1A-serotonin [300], 5-HT4-serotonin [283], μ-opioid [301] and oxytocin receptors [302], between neuropeptide Y receptor and galanin receptor type 2 [303]. There are heterocomplexes between protomers of GPCRs belonging to classes A and C, for example, between the 5-HT2A-serotonin and metabotropic GluR2-glutamate receptors [304,305], as well as between protomers of class A GPCRs and receptors with tyrosine kinase activity, for example, between 5-HT2A serotonin receptor and fibroblast growth factor receptor-1 (FGFR1) [300].

In the case of gonadotropins, there are various models describing the possible role and assessing the contribution of complex formation in the regulation of the functional activity of class A GPCRs and biased agonism. This is illustrated by the dynamic pattern of monomeric and oligomeric forms of LHCGR, their mutual transitions during receptor binding to ligands and during activation [24,26,28,306,307], as well as the existence and specific functional activity of heterodimeric complexes formed by LHCGR and FSHR [14,25,27,29,30,308]. Indirectly, the importance of complex formation for LHCGR is supported by numerous data on the dimerization capacity of FSHR [214,308,309] and TSHR [24,310–313].

7.2 Homodi(oligo)meric Complexes of the LHCGR

As noted above, in the basal state, the main population of LHCGR (about 60%), like FSHR (about 70%), is presented in a monomeric form, which is typical for most GPCRs of class A [26]. Upon activation by an agonist, the proportion of monomeric forms of the receptor increases, although to a small extent, and this is due to the formation of an activated complex in which LHCGR is represented as a monomer. Thus, using cryoelectron microscopy, it was demonstrated that in the activated complex, including hCG-bound LHCGR and the associated G_s protein, which mediates the activation of the AC and cAMP-dependent pathway, the receptor molecule is preferably in the monomeric form [28]. It is important that when LHCGR is activated by a small molecule allosteric agonist that binds to an allosteric site located within the transmembrane channel, the receptor is most likely also in the form of a monomer [28].

Using dual-color photoactivatable dyes and localization microscopy (PD-PALM), it was shown that during the association process, LHCGRs form predominantly homooligomeric, tri- and tetrameric complexes, while the proportion of homodimeric complexes is significantly lower [26]. The authors carried out the association of mutant forms of LHCGR, which were either deprived of the ability to bind a ligand (LHR^{B-}), or lost the ability to couple with the G_s protein and activate AC (LHR^{S-}). This made it possible to assess the role of dimerization and oligomerization in the binding of gonadotropin and the implementation of its regulatory effects to the cAMP level. It was found that with the co-expression of mutant LHCGR protomers, both the processes of hCG binding and activation of cAMP production are realized, which was evidence of the formation of functionally active receptor complexes and, in addition, indicated in favor of *trans*-activation of LHCGR [26]. It is important that other authors, long before this, demonstrated the *trans*-activation mechanism for the mutant form of LHCGR. This form of LHCGR lacked the ability to bind gonadotropin but retained all the molecular determinants required for G protein activation (LHR^{B-}). It formed a complex with an isolated ectodomain of the same receptor, fixed on the outer surface of the membrane using the transmembrane glycoprotein CD8, and the result of this was not only high-affinity binding of hCG to the resulting construct, but also activation of AC [314]. It should be emphasized that in the monomeric state, ligand-bound LHCGR was also able to activate AC, and with high efficiency, but in this case this can only occur through the *cis*-activation mechanism.

The study of the mechanisms of formation of di- and oligomeric LHCGR complexes showed a key role in their formation of interhelical contacts formed by the TM of each protomer, and in the case of different complexes the set of such interactions differed, although to a small extent.

In all three pairs of protomers studied, such as the associated protomers LHR^{B-}, the homodi(oligo)mer LHR^{S-}-LHR^{S-}, and the heterodimer LHR^{B-}-LHR^{S-}, the complexes were stabilized by contacts between helices TM4 and TM1, as well as by contacts TM3-TM3 and TM5-TM5. At the same time, in the case of the formation of the LHR^{S-}-LHR^{S-} homodi(oligo)mer, an additional contribution was made by the TM4-TM4 and TM1-TM5 contacts; in the case of the LHR^{B-}-LHR^{S-} heterodimer, the TM4-TM4, TM6-TM1 and TM2-TM2 contacts were also formed, and in the case of the LHR^{B-}-LHR^{B-} homodimer, the TM6-TM7 contact [26]. The involvement of TMs in the stabilization of homodi(oligo)meric complexes is supported by the results of studying chimeric LHCGRs, including those lacking the ectodomain or its interface with the TMD, which showed the important role of TMs in complex formation [315]. It is important to note that the TMD is involved in the di(oligo)merization of LHCGR and under conditions of its activation by gonadotropin, which binds to LHCGR ectodomain. Conformational changes occurring in the LRR subdomain and hinge region extend to the TMD, which changes the pattern and efficiency of interactions between TM protomers, thereby affecting the stability of the receptor complex [316]. In this case, the influence of the gonadotropin-bound ectodomain of one protomer on the ligand-free ectodomain of another protomer occurs not directly, but through changes induced by hormone binding in the TMD [316].

The involvement of the TMD in the formation of homodi(oligo)meric complexes entails modulation of the binding of G proteins by the receptor, which, in turn, affects the efficiency of activation of G protein-dependent intracellular effectors. An assessment of the role of complex formation in the binding and activation of the $G_{q/11}$ protein showed that the formation of homodi(oligo)meric complexes of LHCGR provides considerable stimulation of the $G_{q/11}$ protein when using hCG, but is not sufficient for its effective stimulation when LHCGR binds to LH [26]. At the same time, gonadotropin-mediated activation of the G_s protein, the effectiveness of which differs for hCG and LH, is carried out mainly through the monomer LHCGR [28]. The formation of a dimeric complex between wild-type LHCGR or constitutively activated mutant LHCGR with mutant LHCGR having the $Asp^{405}Asn$ and $Tyr^{546}Phe$ substitutions, which, despite maintaining binding to the hormone, is not able to mediate its stimulation of the G_s protein and AC, leads to a decrease in the ability of the receptor to activate cAMP-dependent signaling [317]. In this case, AC stimulation, mediated either through the gonadotropin-bound constitutively active or through the wild-type LHCGRs, is reduced to the same extent. In this case, the mutant protomer lacking AC activity functioned as an inverse agonist, stabilizing the inactive state of the receptor. It should be noted, however, that the decrease in the stimulatory effect of gonadotropin on the intracellular cAMP level during dimerization with the inactive mutant LHCGR protomer was expressed to a small extent, and the EC_{50} value for this effect was reduced by half, while the R_{max} did not change at all [317]. An attempt to heterodimerize wild-type LHCGR with the type 3 melanocortin receptor, also functionally coupled to G_s proteins, did not lead to a change in gonadotropin-induced stimulation of AC, which indicates the specificity of the formation of the complex between the mutant LHCGR and wild-type LHCGR protomers [317]. Significant changes in signal transduction also occur in the case of the formation of heterodimeric complexes between LHCGR and the structurally similar FSHR (see below for more details). In this case, both $G_{q/11}$- and G_s-mediated signaling responses change, and the first of them is enhanced during the formation of a heterodimer complex, while the second is enhanced under conditions of its dissociation [25,27]. This suggests a close relationship between the structure of mono- and oligomeric forms of LHCGR and, to a certain extent, the nature of gonadotropin (LH, CG), on the one hand, and the selectivity and efficiency of stimulation of various types of G proteins coupled to LHCGR, on the other.

7.3 Heterodimerization of the LHCGR with the FSHR

With regard to assessing the allosteric effect of complex formation on the activity of LHCGR, studies of heterodimeric complexes between LHCGR and FSHR are of greatest interest, especially since the formation of such heterodi(oligo)meric complexes leads to a significant change in LHCGR activity, including biased activation of intracellular cascades. There is extensive evidence indicating the formation of heterodimers formed by LHCGR and FSHR and their role in the control of steroidogenesis and folliculogenesis [318], although there is still debate on the formation of stable GPCR heterocomplexes for class A receptors under the *in vivo* conditions [319], including for FSHR/LHCGR heterodimerization [200].

In LHCGR/FSHR heterocomplexes, the binding of FSH to FSHR through a *trans*-activation mechanism causes stimulation of the activity of unliganded LHCGR, thereby triggering LH-dependent cascades even in the absence of significant amounts of LH or hCG. The opposite situation may also occur. The functioning of such complexes is critical for the stages of folliculogenesis, including the early stages of antral follicle maturation, which are FSH-dependent. At these stages, both the number of LHCGR and the concentration of LH in the blood are very low and insufficient for efficient androgen synthesis. At the same time, androgens are precursors for estrogens, significant amounts of which are necessary for the growth and development of the antral follicle [320–323]. It is important that at the early stages of antral follicle development, theca cells, which express LHCGR and produce androgens, are not yet differentiated from granulosa cells, which are not LH-competent. It has been suggested that during the pre-

antral stage of folliculogenesis, there are cells that exhibit both theca and granulosa cell properties and are enriched in FSHR, but express only small amounts of LHCGR [324–327]. Since the expression of LHCGR and the level of LH at the preantral stage are not sufficient to ensure the synthesis of such an amount of androgens that is necessary to maintain the high level of estrogens characteristic of this stage, two models of regulatory influences can be realized here. The first model, proposed almost 40 years ago, suggests the development of LHCGR hypersensitivity to hormonal stimulation in the preantral period [328], but no experimental evidence for this has been obtained [200]. In accordance with the second model, confirmed in recent years by many facts, FSHR/LHCGR heterocomplexes function according to the principle of FSH-induced *trans*-activation of LHCGR [25,30,308]. A factor contributing to the formation of such heterocomplexes is the significant predominance of FSHR on the surface of follicular cells at the early antral stage of folliculogenesis, since the ratio of FSHR to LHCGR is approximately 100:1 [329]. This makes FSHR easily accessible to interact with LHCGR and promotes the formation of hetero-oligomeric complexes with a predominance of FSHR.

The following facts support the functional importance of FSHR/LHCGR heterodi(oligo)mers. In women with inactivating mutations in the gene encoding β-LH, folliculogenesis up to the antral follicle stage proceeds normally, and this is accompanied by normal levels of androgen and estrogen production [330,331]. In addition, back in the 1990s, numerous evidence was obtained that FSH preparations are capable of causing the growth and maturation of follicles and the formation of corpus luteum, and also contribute to an increase in ovulatory potential, including in hypophysectomized rats and mice [332–335], and knockout of the gene encoding LHCGR completely prevented these effects of FSH, indicating the requirement of LHCGR for FSH-mediated regulation of certain stages of folliculogenesis [336]. Thus, at the early stages of the antral follicle, LHCGR, to a greater extent than gonadotropins with LH-like activity, is necessary to maintain the androgenic status, ensuring the normal course of these stages of folliculogenesis [29,337].

It should be noted, however, that in mice knockout of the gene for LHCGR, expression of the constitutively active FSHR ensured follicle maturation from antral to preovulatory and provided a phenotype associated with estrogen production, although it did not support ovulation [338]. In addition, in transgenic male mice knockout for the LHCGR gene, the expression of constitutively active FSHR ensured the synthesis of androgen-dependent genes in Sertoli cells and thereby restored, at least partially, spermatogenesis impaired in $Lhr^{-/-}$ mice [339]. All this indicates that high doses of FSH or hyperactivated forms of FSHR are able to partially replace LHCGR-dependent signaling in both the ovaries and testes, preventing a number of disorders of folliculogenesis and spermatogenesis. This should be taken into account when assessing the possible contribution of FSHR/LHCGR heterodimerization to the control of follicle growth and maturation, as well as to the maturation of spermatogenic cells [338].

Clusters combining FSHR and LHCGR on the surface of theca and granulosa cells were first visualized back in 1980, which suggested the possibility of their physical contact or even the formation of FSHR/LHCGR complexes [340]. Subsequently, using BRET, fluorescence correlation spectroscopy, and other approaches, not only the formation of FSHR/LHCGR heterocomplexes was confirmed, but also molecular determinants that may be involved in their stabilization were identified [15,25,30,308]. It was shown that the FSHR/LHCGR heterodimer is stabilized primarily through the interaction of the outer surfaces of TM5, TM6 and TM7, which include a number of allosteric sites in contact with the lipid phase of the membrane. A similar mechanism provides the stabilization of the homodimeric LHCGR complex [26]. At the same time, the formation of homodi(oligo)meric FSHR complexes, along with the TMD, involves regions of the ectodomain [309], which is similar to the mechanisms of the formation of complexes between type C GPCR protomers, where the formation of homo- and heterodimeric complexes occurs involving both extracellular and transmembrane domains [341–343]. In this regard, it cannot be excluded that extracellular regions, including segments of the hinge region of the LHCGR and FSHR protomers, are also involved in the formation of the FSHR/LHCGR complexes.

It should be noted that endogenous regulators of allosteric sites in contact with the lipid phase of the membrane can be membrane lipids, primarily cholesterol and phospholipids, which allows, through changes in the lipid composition of the plasma membrane, to influence the functional activity of GPCRs, as well as their formation of di(oligo)dimensional complexes. On the other hand, the involvement of allosteric sites located on the lateral surface of the TMD in the interaction between protomers in the receptor complex not only changes the conformation and mobility of the TMD and its interfaces with the ectodomain and ICLs, but also shields sites of interaction with membrane lipids and other hydrophobic molecules, capable of specifically binding to such sites. This may provide cause-and-effect relationships between the physicochemical and structural features of the membrane lipid matrix and the functional state of GPCRs, including their ability to form complexes.

The formation of FSHR/LHCGR heterocomplexes influences signal transduction bias by weakening cAMP-dependent signaling pathways and, conversely, enhancing phospholipase pathways leading to activation of intracellular calcium signaling [25,27]. It has been shown that stimulation of heterodimeric human FSHR/LHCGR complexes with both gonadotropins with LH-like activity (LH

or hCG) and FSH leads to a significant attenuation of the stimulating AC signal [25]. On the other hand, the formation of heterocomplexes between LHCGR and FSHR leads to increased signals through $G_{q/11}$ proteins that mediate the activation of PLCβ [27]. Ligand-free FSHR, when associated with gonadotropin-bound LHCGR, significantly increases the latter's ability to activate $G_{q/11}$ protein, releasing the $G\alpha_{q/11}$ subunit, which stimulates PLCβ, generating inositol 1,4,5-triphosphate and, as a consequence, stimulating release of calcium ions from intracellular stores. When ligand-activated LHCGR associates with FSHR, a reorganization of the LH–LHCGR–$G_{q/11}$ protein complex occurs, ensuring more efficient signal transduction along this pathway [27].

Depletion of intracellular calcium reserves by thapsigargin weakens the potentiating effect of heterodimerization on LH-dependent activation of calcium signaling. Along with the $G\alpha_{q/11}$-subunit, during heterodimerization of FSHR/LHCGR, the $G\beta\gamma$-dimer is also involved in LH-induced stimulation of calcium signaling, and its action is most likely realized by opening calcium channels of the plasma membrane, although other mechanisms of their activation are not excluded, which are independent of the $G\beta\gamma$ dimer. A significant contribution of extracellular Ca^{2+} to the effects of LH under conditions of association of LHCGR with FSHR is supported by the weakening of the effect of gonadotropin on calcium signaling with a decrease in the concentration of Ca^{2+} in the extracellular space, as well as the inhibition of this effect by inhibitors of calcium channels of different types. In this regard, it is interesting that in the absence of FSHR, the stimulating effect of LH on calcium signaling is realized mainly through the release of Ca^{2+} from intracellular stores, and not due to its pumping from the extracellular space. This is, in particular, demonstrated by the lack of influence on it by calcium channel blockers, nifedipine and 2-aminoethoxydiphenyl borate (2-APB) [27]. Thus, ligand-free FSHR is a PAM for LH-induced stimulation of $G_{q/11}$ protein, and simultaneously a NAM for hormone activation of the AC signaling system, demonstrating the properties of a biased allosteric modulator.

One of the mechanisms of the modulating effect of FSHR on LH signaling may be a change in the subunit composition of hetero-oligomeric FSHR/LHCGR complexes. There is evidence that in a ligand-free state such complexes can be tetrameric and include three LHCGR molecules and one FSHR molecule, and when bound to LH they are transformed into complexes with a smaller number of LHCGR protomers or even with one such protomer [26,27]. Thus, by replacing LHCGR protomers with FSHR protomers, the effect of negative cooperativity shown for homodimeric LHCGR complexes is blocked [24], which may be the root cause of increased $G_{q/11}$-mediated signaling [27]. The complexes with a FSHR:LHCGR ratio of 3:1 are interesting because in the case of active FSHR complexes, homotrimeric structures are shown, including three FSHR protomers [239]. In other words, at the level of formation of FSHR/LHCGR complexes, another mechanism for the regulation of signal transduction can be demonstrated, based on the different ratio and number of protomers in such complexes.

Currently, heterodimerization of LHCGR with other GPCRs has not been proven, although the formation of such complexes with the membrane estrogen receptor GPER (GPR30), which is known to form heterodimeric complexes with FSHR, cannot be ruled out [318,344,345]. There is evidence of a relationship between the expression of FSHR and LHCGR and the efficiency of GPER-mediated signaling, which, by analogy with FSHR/GPER heterodimers, may indicate the potential for the formation of LHCGR/GPER heterodimers [346,347], but this requires further study. Interestingly, the expression and activity of GPER changes significantly in diabetes mellitus (DM) and cancer [348]. This may indirectly affect gonadotropin signaling or, on the contrary, be one of the consequences of changes in the activity of FSHR- and LHCGR-competent signaling systems.

8. Antibodies to Gonadotropins and LHCGR as Potential Allosteric Modulators of Their Activity

Allosteric effects on LHCGR can be exerted by autoantibodies produced against its antigenic determinants located in extracellular sites. Recently, stimulating autoantibodies to LHCGR were detected in the blood of women with polycystic ovary syndrome and hyperandrogenemia [47]. In this regard, it must be emphasized that inhibitory autoantibodies to FSHR have been found in women with ovarian failure [349,350]. Since LHCGR and FSHR can heterodimerize, autoantibodies to FSHR, like autoantibodies to LHCGR, are characterized by cross-reactivity with respect to the LH- and FSH-dependent cascades. It cannot be excluded that, as in the case of autoantibodies to β-adrenergic receptors [351,352], antibodies to LHCGR create a "regulatory buffer" that allows modulation of powerful activation signals caused by significant changes in the level of gonadotropins during reproductive cycles, or function as a compensatory mechanism that prevents the development of reproductive pathology [42]. But this issue requires additional study.

Synthetic antibodies produced to various epitopes of LHCGR are not only used to assess the functional significance of various regions of the receptor, but are also characterized by a wide range of biological activities, affecting the receptor, including through allosteric mechanisms [45]. Thus, scFv 13B1 antibodies produced against the hinge region of LHCGR were characterized by agonistic activity, and their effect was independent of the hormone and persisted in the case of constitutively active LHCGR forms [45]. Epitope 313–349 was identified in the *C*-terminal

part of the LHCGR hinge region, which was responsible for the ability of antibodies to stimulate cAMP production via LHCGR, and the key role of the sulfated Tyr^{331} residue in the agonistic effect of these antibodies was established [353].

Along with antibodies to receptors, antibodies to LH and hCG, which have different profiles of pharmacological activity, but, as a rule, neutralize the effect of gonadotropins, can have a significant role in the control of LH-dependent cascades [46]. The hCG treatment of men with hypogonadotropic hypogonadism resulted in the production of anti-hCG antibodies, which neutralized the effects of hCG by interfering with LHCGR activation, rendering such therapy ineffective [354,355]. In women, antibodies to endogenous hCG led to decreased fertility and pregnancy loss in the first trimester, when hCG production plays a determining role in fetal development [356,357]. The negative effects of anti-hCG antibodies on reproductive function have been reported in experiments with animals, including monkeys [43,358].

Antibodies developed against equine CG, which has mixed LH- and FSH-like activity, were characterized by a wide range of effects on FSHR and LHCGR, which was due to different epitopes on the α- and β-subunits of the hormone [88,359]. Some of them suppressed the effects of equine hCG on the activity of FSHR and LHCGR, while others, on the contrary, potentiated the FSH-like effects of the hormone. It was extremely interesting that two antibodies that potentiated the FSH-like activity of equine hCG either did not significantly affect or inhibited the LH-like activity of the hormone. This indicates that the same antibodies have different modulatory effects on the LH- and FSH-like effects of equine hCG, functioning as NAMs, PAMs or silent allosteric modulators [88]. Since antibodies to gonadotropins are present in humans in normal and pathological conditions, as shown for antibodies to hCG in humans [360,361], their ability to specifically influence gonadotropin signaling is one of the factors controlling the functions of the male and female reproductive system [44,46]. It must be emphasized that a complex spectrum of biological activity and the ability to modulate the basal and hormone-stimulated activity of FSHR is characteristic of antibodies to FSH, which gives strong grounds to consider antibodies to gonadotropins as one of the endogenous allosteric regulators of LHCGR, FSHR and their heterodi(oligo)mers [30,46].

A study of the role of glycosylation in the production of autoantibodies to equine hCG showed that the deglycosylated form of the hormone did not cross-react with antibodies, while the glycosylated forms, on the contrary, intensively immunoprecipitated with antibodies of different pharmacological activity, which had stimulating, inhibitory or mild modulating effects on FSH-like activity of equine hCG, assessed by progesterone production by Y1 cells derived from a mouse adrenal tumor and stably expressing FSHR, as well as on LH-like activity, assessed by testosterone production by rat Leydig cells [362]. Thus, glycosylation status directly affects the production of antibodies and their ability to biasly regulate gonadotropin signaling.

9. Low-Molecular-Weight Allosteric Regulators of LHCGR

In addition to the orthosteric site involved in the high-affinity binding of gonadotropin molecules, LHCGR contains allosteric sites localized in various loci of the molecule, including in the upper half of the transmembrane tunnel, which remain free when gonadotropin binds [30,42]. It has been established that the transmembrane tunnel of LHCGR contains at least two allosteric sites, the main one and the one modulating its activity [219,363,364]. The first of them, as in a number of other GPCRs, is formed by the internal surfaces of helices TM4, TM5, TM6 and TM7 [363,365,366], while the second is formed by the helices TM1, TM2, TM3 and TM7 [349]. Both sites, the main and modulatory ones, physically overlap each other. The consequence of this overlap, as well as interaction, direct or indirect, with other allosteric sites, is to provide a wide pharmacological range of allosteric regulation of LHCGR. A similar arrangement of allosteric sites in the upper half of the transmembrane tunnel has been postulated for other receptors of pituitary glycoprotein hormones [30,42,367]. In the process of developing LMW ligands of the transmembrane allosteric site of LHCGR, both full and inverse allosteric agonists with intrinsic activity and allosteric modulators were developed, and allosteric modulators, based on their pharmacological activity, can be classified as PAMs and NAMs [30,42]. Along with this, allosteric regulators with ago-PAM activity have been identified [42]. A wide range of LMW allosteric regulators, the targets of which are allosteric sites located in the transmembrane channel, have also been developed for FSHR and TSHR [42,368,369].

9.1 Thienopyrimidine Derivatives as Allosteric Full Agonists and PAMs of LHCGR

The first LMW ligands of LHCGR were developed by Dutch scientists in 2002, who synthesized thieno[2,3-d]pyrimidine derivatives (TPDs) with agonist activity, including the most active compound Org41841 (*N-tert*-butyl-5-amino-4-(3-methoxyphenyl)-2-(methylthio)thieno[2,3-D]pyrimidine-6-carboxamide) and its analog Org43553. Both of these compounds activated LHCGR at nanomolar concentrations, and Org43553 showed higher activity [370]. Subsequently, these compounds became prototypes for a large number of TPDs with the activity of allosteric regulators of both LHCGR and TSHR [42,371–384]. We, based on Org43553 as a prototype of LMW ligands of LHCGR, have developed a series of TPDs with LHCGR agonist/ago-PAM activity, including the *in vivo* active compounds 5-amino-N-tert-butyl-4-(3-(is

onicotinamido)phenyl)-2-(methylthio)thieno[2,3-d]pyrimidine-6-carboxamide (TP01), 5-amino-N-(tert-butyl)-2-(methylthio)-4-(3-(thiophene-3-carboxamido)phenyl)thieno[2,3-d]pyrimidine-6-carboxamide (TP02), 5-amino-N-tert-butyl-2-(methylsulfanyl)-4-(3-(nicotinamido)phenyl)thieno[2,3-d]pyrimidine-6-carboxamide (TP03), 5-amino-N-tert-butyl-4-(3-(1-methyl-1H-pyrazole-4-carboxamido)phenyl)-2-(methylsulfanyl)thieno[2,3-d]pyrimidine-6-carboxamide (TP4/2), 5-amino-N-(tert-butyl)-4-(3-(2-methoxynicotinamido)phenyl)-2-(methylthio)thieno[2,3-d]pyrimidine-6-carboxamide (TP21), and 5-amino-N-tert-butyl-4-(3-(2-chloronicotinamido)phenyl)-2-(methylthio)thieno[2,3-d]pyrimidine-6-carboxamide (TP23) [379,380,383–386] (Table 5, Ref. [363,370,373,375,376,379,380,383–403]).

When studying the compound Org43553, it was shown that it specifically binds to LHCGR (K_d, 2.4 nM), and in its presence, the binding of gonadotropins to the orthosteric site of LHCGR and their stimulating effect on AC activity is preserved, which indicates a mismatch in the localization of the allosteric and orthosteric sites [404]. The result of binding of Org43553 to LHCGR in cells where LHCGR was expressed was stimulation of AC and cAMP-dependent transcription factor CREB with EC_{50} values of 28 and 4.7 nM, respectively, although the effectiveness of Org43553 was lower compared to LH [387]. The compounds we developed, TP01, TP02, TP03, TP4/2, TP21 and TP23 stimulated AC activity in fractions of plasma membranes isolated from the testes and ovaries with moderate efficiency, and did not significantly affect the AC-stimulating effects of hCG, and their effects were suppressed by cholera toxin, the substrate of which are G_s proteins [379–381,383,385,405,406] (Fig. 4). The most active in the *in vivo* conditions were TP03 and TP4/2, which contained pyridin-3-yl and 1-methyl-1*H*-pyrazol-4-yl groups in a variable part, and according to the results of molecular docking, they most effectively interacted with the transmembrane allosteric site of LHCGR [383,384,386]. Both compound Org43553 and compounds TP03 and TP4/2, when exposed to primary cultures of rodent Leydig cells, increased the intracellular level of cAMP and enhanced steroidogenesis, which led to a dose-dependent increase in testosterone production [375,384].

Despite the fact that Org43553 and the TPDs we studied were significantly inferior to LH and hCG in terms of binding affinity and AC-stimulating effect, their lower affinity and moderate AC stimulation are more a positive factor than their disadvantage, because it does not lead to the hyperactivation of cAMP-dependent cascades and prevents the resulting side effects of gonadotropins. The Org43553, TP01, TP03, TP4/2 and TP23 had little or no effect on FSHR and TSHR activity, suggesting their receptor specificity for LHCGR [381,383,385,387,407]. This distinguishes them from the compound Org41841, which, although to a small extent, interacted with the transmembrane allosteric site of TSHR, which subsequently made it possible to create on its basis a wide range of allosteric TSHR regulators with different profiles of pharmacological activity [371,372,374,377,378].

As mentioned above, LH, through $G_{q/11}$ proteins, stimulates the activity of PLCβ, and activation of this enzyme requires higher concentrations of gonadotropin than activation of AC [8,15,408]. Compound Org43553 and the TPDs we developed in the micromolar concentration range stimulated AC both in cell cultures and in plasma membrane fractions of LH-competent tissues, while weakly affecting the activity of $G_{q/11}$ proteins, PLCβ and phosphoinositide metabolism [380,385,387] (Fig. 5). It was shown that Org43553, even at relatively high concentrations (10^{-6}–10^{-5} M), stimulated PLCβ activity by only 33–37%, which is less than 5% of the corresponding effect of LH [387]. There is reason to believe that Org43553 also weakly affects the activity of β-arrestins, which are key targets of gonadotropins, as illustrated by the lack of a pronounced effect on components of the MAPK cascade and the low intensity of LHCGR endocytosis [387]. It is also very important that TPDs, unlike gonadotropins, even when applied in a course in the *in vivo* conditions, have a relatively weak effect on the expression of the gene encoding LHCGR and on the density of LHCGR in target cells, which prevents resistance to the action of endogenous gonadotropins with LH-activity [383,384,409].

Site-directed mutagenesis and molecular docking of Org41841, Org43553 and their analogues were used to reconstruct the transmembrane allosteric site of LHCGR. For this purpose, the AARs forming the allosteric site TSHR, located in the transmembrane tunnel of the receptor, were replaced with LHCGR residues corresponding in localization, as a result of which this TSHR site was structurally close to that of LHCGR [371]. The $Leu^{570}Phe$ substitution in ECL2 TSHR resulted in Org41841 binding to the mutant receptor with an EC_{50} value of 800 nM, while the double substitutions $Leu^{570}Phe/Phe^{585}Thr$ and $Leu^{570}Phe/Tyr^{643}Phe$ resulted in Org41841 binding with an EC_{50} value of 1000 nM. Simultaneous replacement of nine residues ($Ile^{560}Val$ and $Leu^{570}Phe$ in ECL2, $Prp^{577}Thr$, $Ala^{579}Ser$, $Leu^{580}Gln$, $Ala^{581}Val$ and $Phe^{585}Thr$ in TM5, $Tyr^{643}Phe$ and $Ile^{648}Ala$ in TM6) with the corresponding LHCGR residues resulted in a mutant receptor, upon binding to which Org41841 had the activity of full agonist. Its maximum AC stimulating effect was comparable to that of TSH. These data indicate the important role of the $Leu^{570}Phe$, $Phe^{585}Thr$, and $Tyr^{643}Phe$ residues in the formation of the hydrophobic surface of the LHCGR allosteric site located in the transmembrane channel [371]. The key for interaction with Org41841 is the negatively charged residue Glu^{506}, localized in TM3 and highly conserved in GPCRs. Its replacement with alanine blocked the binding of Org41841 to mutant LHCGR and prevented its AC activation. The side carboxylate of Glu is proposed to form a salt bridge with the positively charged amino group

Table 5. Low-molecular-weight allosteric regulators of luteinizing hormone/chorionic gonadotropin receptor (LHCGR): chemical structure, pharmacological profile and efficacy.

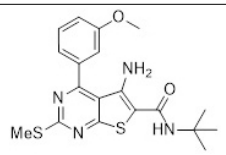
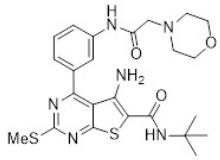
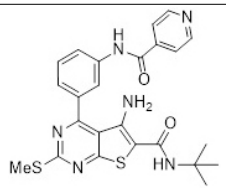
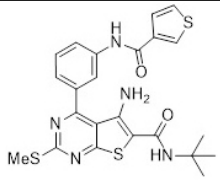
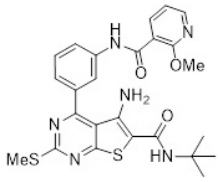
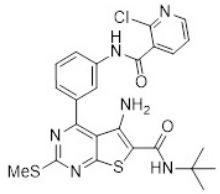
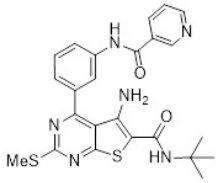
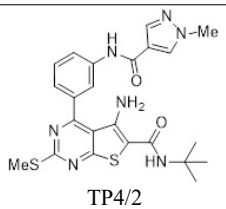
Compound	Efficiency	Structure
Full allosteric LHCGR agonists and/or ago-PAMs		
Thieno[2,3-d]-pyrimidine derivatives (Org41841, Org43553) [370,373,375,376,387–389]	Stimulate G_s proteins and AC in cells with expressed LHCGR; when administered to male rats, they increase testosterone levels; when administered to female rats and female volunteers, they induce ovulation. There is no competition with gonadotropins for binding to LHCGR	 Org41841
		 Org43553
Thieno[2,3-d]-pyrimidine derivatives (TP01, TP02, TP21, TP23) [379,380,385]	Stimulate the activity of G_s proteins and AC in testicular and ovarian membranes, increase testosterone production when administered intraperitoneally and orally to male rats	 TP01
		 TP02
		 TP21
		 TP23
Thieno[2,3-d]-pyrimidine derivatives (TP03, TP4/2) [383,384,386,390–393]	Stimulate the AC system in testicular membranes; activate steroidogenesis and testosterone production in cultured Leydig cells; stimulate testicular steroidogenesis when administered intraperitoneally, subcutaneously and orally to male rats, including those with diabetes mellitus (DM), and aging animals. Activate ovarian steroidogenesis and cause induction of ovulation in mature and immature female rats. Potentiate the steroidogenic effects of hCG in the *in vitro* and *in vivo* conditions	 TP03
		 TP4/2

Table 5. Continued.

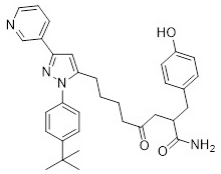
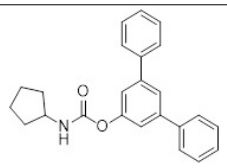
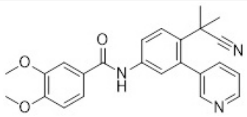
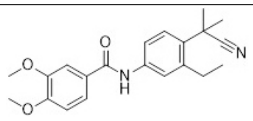
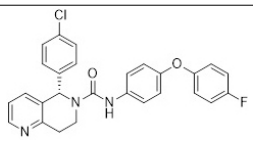
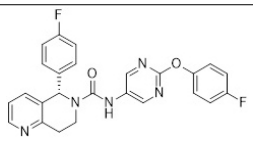
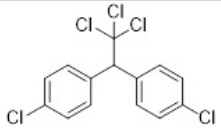
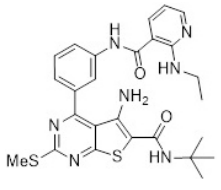
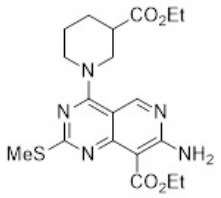
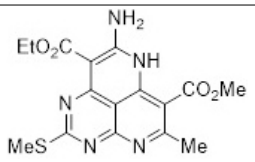
Compound	Efficiency	Structure
Pyrazole derivative, Compound 1 [394]	Stimulates AC activity and testosterone production in Leydig cells	
Pepducin 562–572-K(Palm)A, and its dimeric analogue, including cross-linked regions 562–572 [395,396]	Stimulate AC and G_s proteins in the testicular and ovarian membranes of rats. Peptide 562–572-K(Palm)A increases testosterone production in male rats when administered intratesticularly	Asn-Lys-Asp-Thr-Lys-Ile-Ala-Lys-Lys-Nle-Ala$^{562-572}$-Lys(Palm)-Ala-amide; (Asn-Lys-Asp-Thr-Lys-Ile-Ala-Lys-Lys-Nle-Ala$^{562-572}$)$_2$-Lys-amide
Allosteric LHCGR antagonists and/or NAMs		
Terphenyl derivative, LUF5771 [363,397]	Inhibits the stimulatory effects of hCG and allosteric agonists on LH-dependent intracellular cascades	
Benzamide derivatives (ADX68692, ADX68693) [398]	They inhibit the activity of LH-dependent signaling cascades stimulated by gonadotropins. The specificity of the effects in different cell types and with different ratios of LHCGR and FSHR expression has been shown	 ADX68692  ADX68693
Tetrahydro-1,6-naphthyridine derivatives (BAY-298, BAY-899) [399]	Reduce the production of steroid hormones stimulated by gonadotropins and allosteric agonists; inhibit testicular and ovarian steroidogenesis; suppress folliculogenesis when administered to female rats	 BAY-298  BAY-899
Dichlorodiphenyl trichloroethane [400,401]	Reduces the stimulating effect of hCG on AC, and suppresses LH/hCG-induced recruitment of β-arrestins. Does not significantly affect gonadotropin-stimulated testosterone production, indicating that it selectively acts on different signaling cascades	
NAMs and/or inverse allosteric LHCGR agonists		
Thieno[2,3-d]-pyrimidine derivative TP31 [402,403]	Reduces the stimulating effects of hCG and TP03 on AC activity in testicular membranes (to a greater extent in the case of TP03 stimulation), and inhibits hCG-induced stimulation of testosterone production in male rats. Induces a slight decrease in basal testosterone levels	

Table 5. Continued.

Compound	Efficiency	Structure
Pyrido[3,4-*d*]pyrimidine derivative (PP17) and pyrimido[4,5,6-de][1,6]naphthyridine (PP10) derivatives [402]	Inhibits the stimulatory effects of hCG and TP03 on the AC system in testicular membranes (more so in the case of hCG), and inhibits hCG-stimulated and basal testosterone levels in the blood of male rats	 PP17  PP10

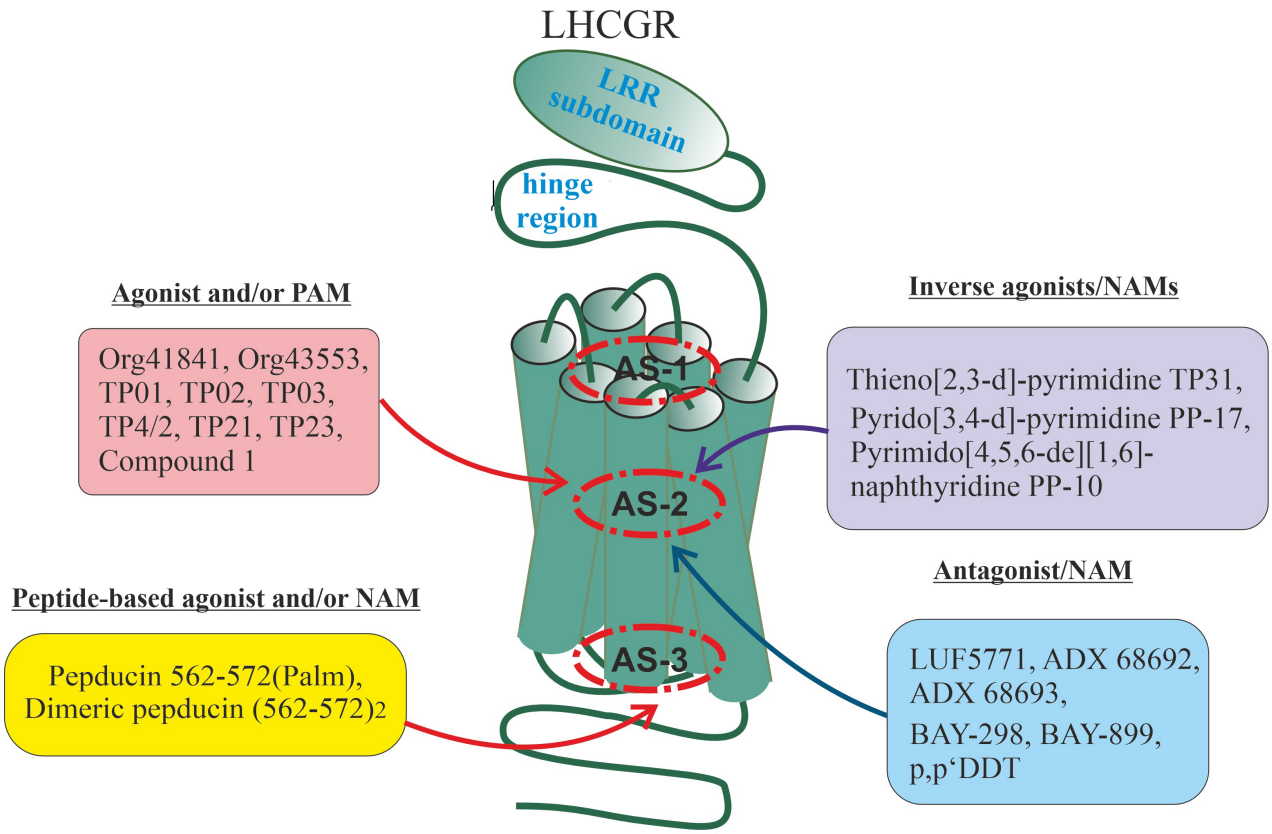


Fig. 4. Synthetic allosteric regulators of luteinizing hormone/chorionic gonadotropin receptor (LHCGR) with different pharmacological activity profiles. The localization of allosteric sites in LHCGR and their low-molecular-weight ligands with different pharmacological activity profiles, including pepducins, which are palmitoylated fragments of the third cytoplasmic loop of LHCGR, are shown. For designations and chemical formulas for the compounds shown in the figure, see Table 5. AS-1, AS-2 and AS-3: allosteric sites in the loci 1, 2 and 3.

of the low molecular weight ligand, ensuring its efficient binding to the receptor [371,372].

In 2021, Duan *et al.* [28] examined the complex of Org43553 with the transmembrane allosteric site LHCGR using cryo-electron microscopy and showed that this small molecule agonist occupies the upper part of the pocket formed by this site. In this case, the morpholine ring of Org43553 is directed outward, to the boundary between the hinge region and the extracellular part of the TMD, while the tert-butylamine group is directed deep into the transmembrane tunnel, to the place where the orthosteric site is located in most class A GPCRs. The allosteric site includes amino acid residues belonging to helices TM3, TM5, TM6 and TM7, loops ECL2 and ECL3 and the segment

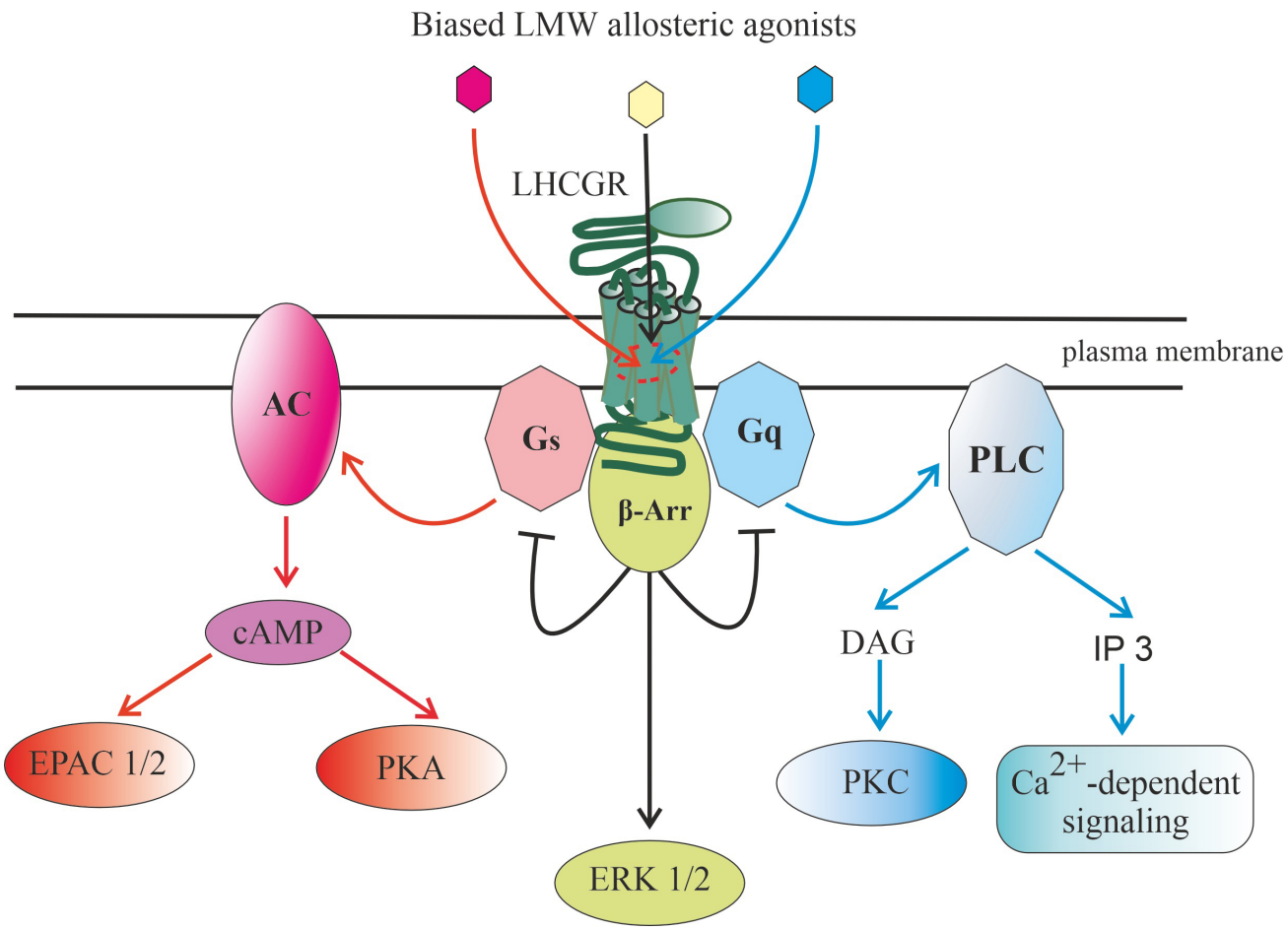


Fig. 5. Regulation of luteinizing hormone/chorionic gonadotropin receptor (LHCGR) activity by biased low-molecular-weight allosteric agonists. A characteristic feature of allosteric agonists is a bias in the activation of intracellular signaling cascades, which is clearly shown for thienopyrimidine derivatives (TPDs) such as Org43553, TP03 and TP4/2, which activate cAMP-dependent signaling pathways with high selectivity. The figure shows various variants of biased signaling, when an allosteric agonist preferentially activates either the adenylyl cyclase signaling cascade (*red arrows*), or phospholipase C and calcium signaling (*blue arrows*), or β-arrestins and processes dependent on them (desensitization and down-regulation of the receptor, β-arrestin endosomal signaling) (*black arrows*, including those blocking G protein-mediated signaling). The bias of allosteric agonists may be due to the characteristics of their chemical structure when binding to the same allosteric site, or the ability to bind to topologically different allosteric sites. *Abbreviations*: AC, adenylyl cyclase; G_s, heterotrimeric G proteins that stimulate adenylyl cyclase activity; PKA, protein kinase A; EPAC 1/2, Exchange Protein directly activated by Cyclic AMP, types 1 and 2; PLC, phosphoinositide-specific phospholipase Cβ; G_q, heterotrimeric G proteins that and stimulate PLCβ and calcium signaling; DAG, diacylglycerol; IP3, inositol-3,4,5-triphosphate; PKC, protein kinase C; ERK 1/2, extracellular signal-regulated kinases, types 1 and 2, the effector components of mitogen-activated protein kinase cascade; LMW agonist, low-molecular-weight agonist.

of the hinge region bordering TM1, and substitutions of two of them, Ala^{589}Trp and Ile^{585}Phe, significantly reduce the agonistic activity of Org43553, despite maintaining the affinity of this TPD to the receptor. The importance of hydrophobic interactions between Org43553 and the transmembrane allosteric site of LHCGR has also been shown [28].

Previously, using homology modeling methods and molecular dynamics, we assessed the binding parameters of TP4/2 and other TPDs developed by us with LHCGR and also showed that hydrophobic interactions play a decisive role in stabilizing the complex of a LMW agonist with a transmembrane allosteric site, while Coulomb interactions and hydrogen bonds were less significant [383]. As in the case of Org43553, the 1-methyl-1H-pyrazol-4-yl group was directed to the extracellular entrance of the transmembrane tunnel, and the *tert*-butylamine group to the central part of this tunnel. A reduced ability of TP4/2 to interact with the transmembrane allosteric site of TSHR was also shown, which is in agreement with the data on the absence of a significant effect of this compound and its analogues on TSH-stimulated AC activity in the membranes of the thy-

roid gland, and *in vivo* on the levels of thyroid hormones and the expression of genes responsible for their synthesis [383,385,407].

9.2 The In Vivo and Clinical Effects of Thienopyrimidins Derivatives with Agonistic Activity

It is important that Org43553 and the TPDs we developed are active not only *in vitro*, but also *in vivo*, and they are effective both in parenteral (intraperitoneal, subcutaneous) and oral routes of administration [375,376,379,381,383–386,388,389]. Retention of activity upon oral administration indicates good absorption of TPDs in the gastrointestinal tract and their high bioavailability with this method of delivery. The oral bioavailability of Org43553 was 79% in rats and 44% in dogs compared to parenteral bioavailability [375]. We demonstrated comparable bioavailability and effectiveness in stimulating steroidogenesis with intraperitoneal and oral delivery methods for compounds TP03 and TP4/2 [379,383,384]. A single oral administration of Org43553 at a dose of 50 mg/kg caused ovulation in immature mice and mature rats, and the resulting oocytes were of good quality, characterized by high fertility, normal implantation of fertilized eggs into the uterus, and a high yield of viable embryos [375]. A single oral administration of Org43553 at the same dose to male rats stimulated testicular steroidogenesis and increased blood testosterone levels [375]. Both oral and intraperitoneal administration of TP03 and TP4/2 increased testosterone production in male rats, and this effect was maintained during a course of drug administration for up to 5 days or more [383,384]. Various routes of administration of TP03 and TP4/2, including oral administration, induced ovulation in immature female rats [386,392]. Since oral administration is preferred and is especially valuable when using LHCGR agonists in assisted reproductive technologies, the comparability of bioavailability and physiological response between oral and parenteral delivery routes of TPDs is one of their advantages over gonadotropins, which can only be administered parenterally.

When TP4/2 and TP03 were administered to male rats for five days, they showed a moderate, sustained increase in blood testosterone levels [383,384]. In contrast to hCG, the steroidogenic effect of TPDs did not change significantly during the treatment period, while in the case of gonadotropin it reached a maximum in the first two days, decreased significantly on the third day (below the level of treatment with low molecular weight agonists) and on days 4–5 was comparable to that of TPDs [383,384]. This is due to the fact that TPDs do not suppress the expression of the gene encoding LHCGR and have little effect on its distribution in the testes, while gonadotropins, especially hCG, sharply reduce both the expression of this gene and the density of LHCGR in the testes, inducing Leydig cells resistance to LH [383,384,409]. Despite the fact that TP03 and TP4/2 more mildly, in comparison with hCG, stimulated the expression of the main genes of testicular steroidogenesis (StAR, P450-17α), these effects persisted with long-term administration of the drugs, while in the case of hCG they were weakened [383,384]. In TP03-treated rats, there was an increase in the proportion of sperm with progressive movement, as well as an increase in the number of spermatogonia and pachytene spermatocytes in the seminiferous epithelium, indicating stimulation of spermatogenesis by LMW agonists of LHCGR [384].

The testicular steroidogenesis- and spermatogenesis-stimulating effects of TPDs have been demonstrated not only in healthy male rats, but also in aging animals, in rats with the types 1 and 2 DM, and with diet-induced obesity, all of which had varying degrees of severe androgen deficiency and impaired spermatogenic function, leading to decreased fertility [383,384,410].

When administered to male rats with streptozotocin type 1 diabetes and aging (18 months) animals, TP4/2 restored their androgenic status, normalized the thickness of the germinal epithelium, the number of spermatogonia and spermatocytes, thereby restoring impaired spermatogenesis [383]. TP03 compensated for androgen deficiency and restored attenuated spermatogenesis in male rats with type 2 DM induced by a high-fat diet and low dose of streptozotocin [384]. Despite some weakening of the steroidogenic effects of single-administered TPDs in the types 1 and 2 DM and aging compared with those in control animals, it was less pronounced than in the case of hCG and was absent with long-term administration of TPDs. This may be due to the preservation of the amount of functionally active LHCGR in the testes, which we assessed immunohistochemically [383,384]. Five-week treatment of male type 2 DM rats with metformin partially restored testicular steroidogenesis and spermatogenesis and enhanced the steroidogenic effects of TP03 and hCG, indicating improved testicular sensitivity to both allosteric and orthosteric LHCGR agonists in metformin-treated animals. However, with long-term administration of TP03 and hCG, their steroidogenic effects during metformin therapy, on the contrary, were weakened, which may be a compensatory reaction to hyperactivation of the testicular steroidogenesis system under the combined influence of metformin and LHCGR agonists [384,411]. This opens up prospects for the development and optimization of approaches to correct androgen deficiency, which is based on metabolic disorders, using combination therapy, including allosteric LHCGR agonists.

Pharmacokinetic studies have shown that Org43553 is degraded faster than gonadotropins [375]. Thus, in rats, the half-life of Org43553 was about 3 hours, while for hCG it reached 6–7 hours. Our assessment of the half-life of TP03 and TP4/2 showed similar values for both oral and intraperitoneal routes of administration (Shpakov, Derkach, *unpublished data*). Reducing the half-life is of great practical importance because it reduces the activation time of LHCGR,

and this prevents hyperactivation of LH-dependent cascades and prevents the development of resistance to endogenous gonadotropins. As a result, the risk of developing ovarian hyperstimulation syndrome, one of the most serious complications during controlled ovulation induction using hCG and LH, is reduced, and the potential oncogenic risks associated with the use of gonadotropins in the correction of androgen deficiency in men are also reduced.

With a single treatment of mature rats with hCG, a significant increase in the size of the ovaries, an increase in vascular permeability, and hypersecretion of vascular endothelial growth factor (VEGF) by granulosa cells are observed, which are characteristic signs of ovarian hyperstimulation syndrome. At the same time, when Org43553 was administered to female rats, the size of the ovaries and vascular permeability in them changed to a small extent, and even repeated treatment of animals with this agonist did not cause ovarian hyperstimulation syndrome [376]. One reason for this is the weak effect of Org43553 on the production of VEGF, a potent angiogenic factor and inducer of increased vascular permeability [376]. When studying orally administered TP4/2 (40 mg/kg) to immature female rats stimulated two days earlier with Follimag, it was shown that TP4/2 time-dependently stimulates the production of progesterone and the formation of the corpus luteum, increases the expression ovarian genes responsible for steroidogenesis and involved in the control of ovulation, and also increases the gene expression and amount of the ADAMTS metalloproteinase, the most important marker of ovulation [386,392]. All these effects had some similarity to those of hCG, but were less pronounced, indicating a moderate stimulation of ovulation by the LMW agonist of LHCGR. There were significant differences between hCG and TP03 regarding the stimulation of gene expression of the proangiogenic factor VEGF type A (VEGF-A). If, after treatment with hCG, the level of expression of the VEGF-A gene in the ovaries remained elevated throughout 24 hours after treatment with gonadotropin in comparison with the group treated only with Follimag, while in the case of TP03 it was increased only after 4 hours, and then decreased to the control level [386]. It should be noted that increased levels of VEGF in the tissues of the reproductive system are a trigger for the development of malignant neoplasms and contribute to the metastasis of existing tumors. As a result, LMW agonists of LHCGR are believed to have low pro-tumorigenic potential, which, however, requires further study.

Successful animal experiments allowed for clinical trials of Org43553 as an ovulation inducer in women [389]. After oral administration of Org43553 at the doses from 25 to 900 mg, its peak concentration was reached after 0.5–1 hour, and the half-life was 30–47 hours. At a dose of 300 mg, Org43553 induced ovulation in 83% of women of reproductive age, without causing significant side effects. effects. There were no signs of ovarian hyperstimulation syndrome when taking Org43553 in female volunteers [389]. Compound Org43553 is patented by Organon/Merck & Co/Merck Sharpe & Dohme (MSD) (Kenilworth, USA), its main developer [373]. The ability of this compound and other TPDs with LHCGR agonist activity to increase testosterone levels in men can be used to compensate for androgen deficiency, correct hypogonadotropic conditions, and also to increase muscle size and strength in athletes when using TPDs as anabolic steroids. In this regard, Org43553 and its analogues were recommended for inclusion in the list of controlled substances in doping tests already at an early stage of their development [412].

9.3 Additivity of Steroidogenic Effects of Thienopyrimidines and hCG and Possible Chaperone-Like Effects of Thienopyrimidines with LHCGR Agonistic Activity

One of the approaches to prevent complications of gonadotropin therapy is to reduce their dose, but this, as a rule, leads to a significant weakening of the effectiveness of the drugs and failure to achieve the required effect, including assisted reproductive technologies. We and other authors have demonstrated the partial additivity of the effects of low doses of gonadotropins and allosteric LHCGR agonists on AC activity and testosterone production in the *in vitro* conditions [385,387]. This made it possible to formulate a hypothesis about their additivity and potentiation under the *in vivo* conditions. Such effects are due to both different localization of orthosteric and allosteric sites in the LHCGR (additivity) and the mutual influence of these sites (potentiation). In the first case, TPDs can act as a full allosteric agonist; and in the second case, they can function as PAM or ago-PAM. In support of this, we showed that pretreatment of male rats with TP03 in doses from 7.5 to 25 mg/kg (i.p.) almost doubled the steroidogenic effect of hCG and reduced its effective dose, and also significantly changed the hCG-stimulated expression pattern steroidogenic genes [413]. There is evidence that gonadotropin is able to facilitate the binding of LHCGR to a LMW agonist. This is based on a change in the interaction of the LRR subdomain and hinge region with the extracellular loops and the outer vestibule of the TMD after receptor binding to gonadotropin. The result is a change in the superposition of TM6 and TM7 and an increase in the volume of the transmembrane tunnel cavity in which the allosteric site of LHCGR is localized, and this changes its accessibility and affinity for the LMW ligand [95].

Along with the mutual influence of the orthosteric and allosteric sites, an important contribution to the potentiation effect can be made by the chaperone properties inherent in allosteric LHCGR agonists, which can be most pronounced in the case of mutant forms of the receptor with a reduced ability to translocate into the plasma membrane (Fig. 6). LHCGR with Ala^{593}Pro and Ser^{616}Tyr mutations in the TMDs are not capable of translocation into the plasma membrane and remain in the endoplasmic reticulum, remaining in an inactive state, despite retaining the ability to

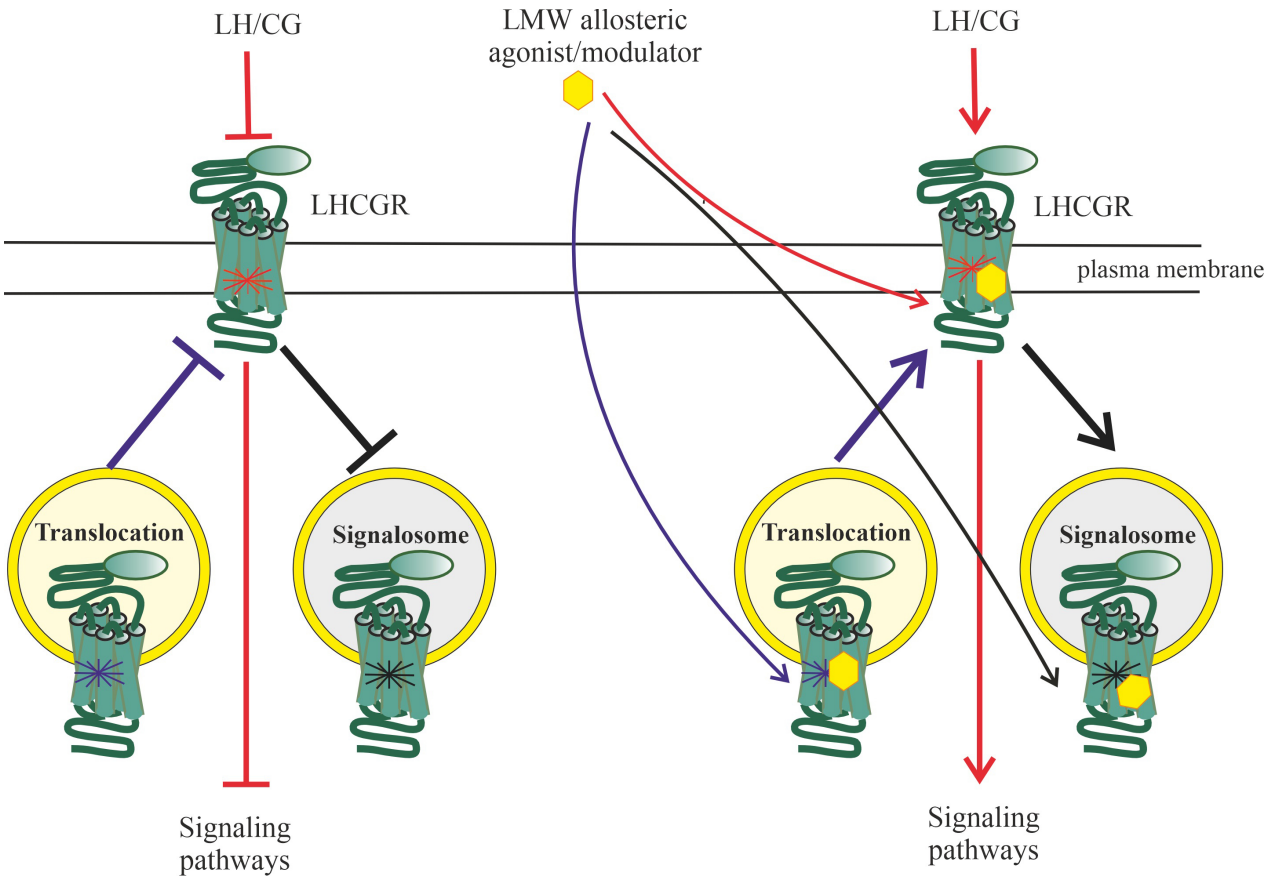


Fig. 6. Chaperone-like activity of low-molecular-weight (LMW) ligands of luteinizing hormone/chorionic gonadotropin receptor (LHCGR) allosteric sites. Mutations in LHCGR can lead to a decrease in the affinity of the receptor for gonadotropin and weakening of gonadotropin-induced receptor activation (red blocking arrow), deterioration of post-translational processing of the receptor and its translocation into the plasma membrane (purple blocking arrow), as well as disruption of interaction with β-arrestins and prevention of the endosomal signaling (black blocking arrow). An allosteric LMW agonist or modulator is capable of binding to allosteric sites of LHCGR located on the cell surface (direct interaction of receptor with an extracellular allosteric ligand), as well as to LHCGR located in intracellular compartments, which is due to the ability of a hydrophobic allosteric ligand to penetrate through the plasma membrane into the target cell. Binding of the mutant LHCGR to a suitable allosteric ligand leads to stabilization of the receptor conformation, similar to that of the wild-type LHCGR, which can ensure normal binding of the allosteric ligand-bound mutant LHCGR to gonadotropin and its activation (red arrow), normalize translocation of the mutant receptor into the membrane (purple arrow), as well as ensure the formation of the signalosome responsible for intracellular LHCGR-mediated signal transduction (black arrow). Thus, a suitable allosteric ligand can act as an extracellular and/or intracellular LMW chaperone for the mutant receptor, which is in both a free and gonadotropin-bound state. Potentially, such chaperones can be either hydrophobic allosteric ligands of allosteric sites located inside the receptor transmembrane channel, or pepducins (peptides modified by fatty acid radicals that are derivatives of the receptor intracellular loops), which are able to penetrate the membrane into the cell and specifically interact with the cytoplasmic allosteric sites of the receptor.

bind to gonadotropins. Mutant receptors have been identified in patients with reproductive dysfunctions caused by hypoplasia of testosterone-producing Leydig cells [414–417]. The compound Org42599 with LHCGR agonist activity, which is a trifluoroacetate salt of Org43553, restores the activity of mutant LHCGR with Ala^{593}Pro and Ser^{616}Tyr substitutions [418]. Incubation of cells expressing mutant LHCGR with Org42599 increased expression of the mutant receptor, the proportion of LHCGRs with normal folding of the polypeptide chain and suitable topology in the membrane, and also increased the density of LHCGRs on the cell surface. This effect was associated with the ability of Org42599 to penetrate the plasma membrane of Leydig cells and specifically bind to the allosteric site of the receptor located in the reticular membrane, which ensured the correct folding of LHCGR and its translocation into the membrane [418]. The transition of LHCGR to the active state prevented its degradation in proteasomes, which was due to a change in the interaction of the mutant receptor in the Org42599-bound state with enzymes and chaperone proteins responsible for the maturation, translocation and degradation of LHCGR. The mutant LHCGR in complex

with Org42599, like the normal receptor, was able to be modified by protein disulfide isomerase, which catalyzes the formation of disulfide bonds in proteins and, thereby, ensuring their appropriate folding. Along with this, mutant LHCGR in complex with Org42599 lost the ability to form complexes with 94 kDa glucose-regulated protein (Grp94) and Ig-binding protein (BiP), which transport misfolded proteins to the site of their degradation in proteasomes [418].

The chaperone-like effect of TPDs may be mediated not only by the potentiating effect of these compounds on the effects of gonadotropins *in vitro* and *in vivo*, but also by the high efficiency of TPDs, including TP03, in stimulating steroidogenesis in rats with the types 1 and 2 DM. Moderate and severe forms of type 1 DM in humans, as well as in animals with experimental models of type 1 DM, are characterized by severe androgen deficiency and impaired sensitivity to gonadotropins, as demonstrated by us and other authors [33,419–422]. The reasons for this are an increase in the content of reactive oxygen species, a weakening of the antioxidant defense system and the intensification of inflammatory and apoptotic processes in the testes, as well as structural changes in testicular tissue [423–426]. Based on the above, it can be assumed that when diabetic rats are treated with TPDs, these compounds, being hydrophobic substances, penetrate the plasma membrane and stabilize intracellularly localized LHCGR in a conformation resistant to degradation, allowing their translocation into the membrane. Thus, the chaperone-like activity inherent in TPDs ensures their restorative effect on androgen deficiency in conditions of metabolic disorders that negatively affect the activity of LHCGR, including due to disturbances in its post-translational modifications, and also in the case of mutant LHCGR with reduced sensitivity to gonadotropins.

9.4 The Other Low-Molecular-Weight Allosteric Regulators of LHCGR

Along with TPDs, other allosteric LHCGR ligands with heterocyclic structures have been developed that have full agonist or PAM activity. Of greatest interest among them are pyrazole derivatives, including Compound 1. It stimulated AC activity (EC_{50}, 20 nM) and increased testosterone synthesis by Leydig cells (ED_{50}, 1.31 µM) in the in vitro conditions, and its effectiveness was comparable to that of hCG [394]. Under the *in vivo* conditions, when administered intraperitoneally to male rats, Compound 1 (32 mg/kg) increased the level of testosterone in the blood of animals. Like Org43553, the pyrazole derivative did not compete with the labeled hormone [^{125}I]-hCG for binding sites with LHCGR. This indicates the interaction of pyrazole derivatives with the allosteric site of the receptor, which does not overlap with the orthosteric site of LHCGR and is probably localized in its transmembrane tunnel [394].

Along with full agonists and/or PAMs, LMW allosteric ligands for LHCGR with the activity of NAMs, neutral antagonists or inverse agonists have been developed, including the derivatives of terphenyl [363, 397], tetrahydro-1,6-naphthyridine [399], pyrimido[4,5,6-de][1,6]naphthyridine, pyrido[3,4-d]pyrimidine [403], benzamide [398] and thieno[2,3-d]-pyrimidine [402,403], as well as dichlorodiphenyltrichloroethane [400,401].

Among terphenyl derivatives, the compound LUF5771 suppressed LHCGR stimulation by both gonadotropins and LMW agonists. LUF5771 at a concentration of 10 µM accelerated the rate of dissociation of [^{3}H]Org43553 from LHCGR by 3.3 times, and also reduced the stimulating effects of hCG and Org43553 on LH-dependent signaling cascades by 2–3 times [363,397]. The study of the molecular determinants responsible for the binding of LUF5771 to the receptor showed that the binding site for LUF5771, as in the case of Org43553 and other TPDs, is localized in the transmembrane tunnel, and partially overlaps with the TPDs-binding site. At the same time, LUF5771-binding site differs from the TPDs-binding site in its set of AARs, including amino acids localized in helices TM1, TM2, TM3, TM6 and TM7, and also in ECL2 [363]. Another terphenyl derivative, compound LUF5419, in the presence of which the inhibitory effects of LUF5771 were reduced by 2–10 times, bound to the same site as LUF5771, with the only difference that the LUF5419-binding site did not include AARs localized in helices TM1 and TM2 [363,397]. There is reason to believe that it is these AARs that may make a decisive contribution to the inhibitory effect of LUF5771 on LHCGR activity.

In 2011, German scientists developed the compounds BAY-298 and BAY-899 based on tetrahydro-1,6-naphthyridine, which have NAMs activity for LHCGR [399]. BAY-298 inhibited testicular and ovarian steroidogenesis stimulated by LH and the allosteric full agonist Org43553, and also impaired follicular maturation when administered to female rats. Importantly, BAY-298 bound to a site distinct not only from the extracellular orthosteric site, but also from the transmembrane allosteric site responsible for binding of Org43553. Like BAY-298, BAY-899 also dose-dependently suppressed the production of steroid hormones when administered to female rats and arrested the cycle in the diestrus and metestrus phases [399].

We have developed two allosteric inverse LHCGR agonists, 8-amino-5-methyl-2-(methylsulfanyl)-6-(methoxycarbonyl)-7*H*-pyrimido[4,5,6-de][1,6]naphthyridine-9-carboxylic acid ethyl ester (PP10), derivative pyrimido[4,5,6-de][1,6]naphthyridine, and 7-amino-4-(3-(ethoxycarbonyl)piperidin-1-yl)-2-(methylsulfanyl)pyrido[4,3-d]pyrimidine-8-carboxylic acid (PP17), a pyrido[3,4-d]pyrimidine derivative [403]. At a concentration of 100 µM, they weakened the AC-stimulating effects of hCG and TP03 in testicular membranes, and when intratesticularly injected into male

rats (10 mg/kg), they inhibited testosterone production. However, when administered intraperitoneally, only PP17 (45 mg/kg) retained the ability to suppress both basal and hCG-induced testicular steroidogenesis, indicating low bioavailability or instability in the bloodstream of PP10 [403].

Additionally, we have developed TPDs with allosteric LHCGR antagonist activity. The most active among them was 5-amino-*N*-(*tert*-butyl)-2-(methylthio)-4-[3-(2-(ethylamino)nicotinamido)-phenyl]thieno[2,3-d]pyrimidine-6-carboxamide (TP31) [402,403]. In the *in vitro* experiments, TP31 (100 μM) effectively inhibited the AC-stimulating effects of hCG and TP03, and in the *in vivo* experiments, when administered intratesticularly to male rats, TP31 (10 mg/kg) reduced the basal level of testosterone in the blood, and its inhibitory effect increased during time, reaching a maximum 5 hours after injection. Intraperitoneal administration of TP31 (45 mg/kg) resulted in a decrease in both basal and hCG-stimulated testosterone levels and prevented the hCG-induced increase in expression of testicular steroidogenec genes [403].

Thus, a series of inverse agonists or NAMs for LHCGR has been developed, both based on TPDs (TP31) and on other heterocyclic compounds (LUF5771, PP17), which can be used to create drugs that reduce the sensitivity of cells of the reproductive system to gonadotropins, which is important for contraception and treatment of gonadotropin- and steroid-dependent tumors.

More recently, it was found that dichlorodiphenyltrichloroethane (p,p′DDT) negatively affects the activity of LHCGR by preventing its activation by gonadotropins [401]. In cultured CHO cells, p,p′DDT reduced the stimulatory effect of hCG on the production of intracellular cAMP and also inhibited the recruitment of β-arrestins induced by LH and hCG. At the same time, p,p′DDT had little effect on testosterone production stimulated by these gonadotropins. Taken together, these data indicate that p,p′DDT functions as NAMs towards LHCGR, and its influence is characterized by a bias towards certain intracellular cascades [401]. It is interesting to note that p,p′DDT is not specific for LHCGR and is capable of influencing FSHR activity, in which case it potentiates its activation by FSHR [400]. Perhaps this dual specificity is due to the ability of FSHR and LHCGR to heterodimerize these receptors.

Another class of compounds that have demonstrated activity against both FSHR and LHCGR are the benzamide derivatives ADX68692 and ADX68693. They inhibited the gonadotropin-stimulated activity of both of these receptors with varying intensity [398]. Quite unexpectedly, the actions of ADX68692 and ADX68693 were specific to both the specific cell types in which FSHR and LHCGR were expressed, and also depended on the expression pattern and ratio of these receptors. This gives grounds to believe that the target of benzamide derivatives was the heterocomplexes of these receptors, which is responsible for their unusual activity profile. Thus, in contrast to allosteric full LHCGR agonists, allosteric neutral antagonists and NAMs are less specific towards LHCGR, and this may be due to both the similarity in the configuration of the "inhibitory" allosteric site of FSHR and LHCGR, and their ability to heterodi(oligo)merization [30,398].

In addition to ligands of the transmembrane allosteric site, ligands of cytoplasmic allosteric sites, including regions involved in interaction with G proteins and β-arrestins, are promising. Hydrophobic LMW heterocyclic compounds can be used as such ligands, for example, parmodulins in the case of proteinase-activated receptors [427,428] and vercirnon and its derivatives for chemokine receptors [429,430]. Also of significant interest are synthetic peptides corresponding to the ICL2, ICL3 regions and the proximal segment of the C-tail cytoplasmic domain of GPCR, as well as structurally including the interfaces of the cytoplasmic loops and TMD, important for signal transduction [431–434]. These peptides, interacting with their complementary regions within the intracellular allosteric site, modulate signal transduction at the stage of receptor coupling with G proteins and β-arrestins, and their action can be highly selective in relation to both the receptor and intracellular cascades. Moreover, they themselves are capable of triggering signal transduction, in the absence of an orthosteric agonist. In order to be available for interaction with an intracellular allosteric site, such peptides must cross the plasma membrane, as a result of which they are modified with a hydrophobic radical, usually a palmitic acid residue (such peptides are called pepducins) [432,433,435–437].

Based on the fact that the *C*-terminal half of ICL3 is involved in interaction with the G_s protein, which indirectly indicates its overlap and/or interaction with the intracellular allosteric G_s-competent site, we synthesized and studied the *C*-terminally palmitoylated peptide NKDTKIAKK-Nle-A(562–572)-K(Pal)A, which corresponded to the region 562–572 of LHCGR, and its analogs [395,396,438,439]. Under the *in vitro* conditions at micromolar concentrations, this peptide increased AC activity and GTP binding in testicular membranes, and when administered intratesticularly to male rats, it stimulated their testicular steroidogenesis and increased testosterone production [395,439]. At the same time, it significantly inhibited the stimulating effects of hCG on the activity of the AC system *in vitro* and on testosterone production *in vivo*, which indicates its pharmacological profile as a PAM antagonist [395,439]. A series of derivatives of peptide 562–572 with acyl radicals of different localization and hydrophobicity was synthesized and it was found that the highest activity was characterized only by those derivatives that had a fatty acid residue at the C-terminus of the peptide, which in native LHCGR borders the intracellular end of the TM6 helix [438]. Thus, it was shown that the hydrophobic radical is necessary not only for the efficient penetration of pepducin

through the membrane, but also for its anchoring on the cytoplasmic side of the membrane, near the site of its interaction with the complementary regions of the receptor ICLs. This provides a suitable position of pepducin for allosteric regulation of LHCGR-mediated signal transduction [438]. It should be noted, however, that when administered subcutaneously and intravenously, the peptide 562–572 LHCGR showed low stability and little activity, which requires further research to improve its resistance to proteolysis.

10. Conclusion and Perspectives

In the process of evolution of vertebrates, along with the complication and improvement of the mechanisms regulating reproductive functions, including a more subtle organization of hormonal control of the estrous cycle in females, there was an increase in the complexity and flexibility of controlling these mechanisms, including at the level of the LH-regulated signaling system. To increase its information capacity and efficiency, the range of signaling cascades and effector proteins activated by gonadotropins with LH activity was expanded, and mechanisms were developed to ensure the targeting of LH signaling and its fine tuning under specific physiological conditions. And to achieve this, various strategies have been implemented.

Some of these strategies were implemented by changing the structure of gonadotropins, endogenous ligands of the orthosteric site of LHCGR, without significantly changing the structure of this site. To this end, at the genome level, through gene duplication and alternative splicing, various forms of gonadotropins were generated, which in humans and some primates are represented by β-LH and β-CG, and, in addition to this, the glycosylation status of gonadotropin subunits at asparagine residues (LH) and at serine or threonine residues (CH, equine LH) also varied significantly. Thus, not only was the efficiency and bias of gonadotropin-induced signal transduction required under certain physiological conditions achieved, but also additional levels of its regulation were provided. First, since LH and CG can be produced by different tissues (pituitary and extrapituitary origin), their expression and secretion in these tissues will be regulated by different factors and by different mechanisms.

Secondly, in different tissues and cell types there are significant differences in the machinery of *N*- and *O*-glycosylation, due to different patterns of expression and activity of glycosyltransferases and glycosidases, which leads to an almost inexhaustible variety of glycoforms of LH and CG. The multiplicity of glycoforms of dimeric gonadotropins is also ensured by the combination of α- and β-subunits of gonadotropins, which have a different set of glycans. In this case, not only the number of glycans plays an important role, but also the degree of their branching and the content and distribution of negatively charged glycosyl residues at the ends of the glycan backbone. It should be noted that a significant, and possibly decisive, contribution to the interaction of both various gonadotropins and their glycoforms with LHCGR is made by allosteric mechanisms. They are due to the fact that the binding of LHCGR to LH or hCG molecules and their various glycoforms can directly or indirectly affect the structural characteristics of allosteric sites localized both in the LRR subdomain, hinge region and ECLs, and in the TMD, as well as on the accessibility these allosteric sites for ligands.

It is also important that this changes the superposition of "internal" allosteric regulators, the functions of which are performed by fragments of the hinge region and, possibly, other parts of the receptor molecule. Direct interaction of *N*- and *O*-glycans of gonadotropins with allosteric sites of LHCGR or glycan-glycan interactions between gonadotropins and the receptor, changing the conformational characteristics of such sites, cannot be excluded.

Other strategies involve "gonadotropin-independent" influence on the conformational characteristics of the LHCGR and its ability to interact with gonadotropin, and these strategies rely almost exclusively on allosteric mechanisms. Firstly, this is the homodi(oligo)merization of LHCGR or the formation of its heterocomplexes with other receptors, primarily with FSHR. The formation of receptor complexes can lead to a weakening or even prevention of the gonadotropin signal (LHCGR homodimerization), and to a certain extent determines its bias towards intracellular targets. In the case of the LHCGR/FSHR heterocomplex, due to *trans*-activation, LH-dependent intracellular cascades can be triggered by FSH. Combining the allosteric effects of the formation of LHCGR complexes on LH/CG-mediated transduction with those of various combinations of gonadotropin subunits and their glycoforms leads, on the one hand, to an even greater variety of potential effects of gonadotropins in the target cell, and on the other hand, allows for finely regulated selectivity and the intensity of the hormonal signal, which is of great importance for the development of drugs with LH-like activity for use in reproductive medicine. Little studied, but of considerable interest, is the possible impact of autoantibodies developed against molecular determinants localized in the ectodomain of LHCGR on its activity, since such autoantibodies, interacting with LHCGR, are capable of both changing its basal (constitutive) LHCGR activity and modulating the effects endogenous gonadotropins on LHCGR, as shown for anti-TSHR antibodies.

The presence of allosteric sites in LHCGR, localized in the TMD and ICLs, creates good opportunities for the development of artificial allosteric regulators of different chemical nature with a wide range of pharmacological activity. Currently, the main emphasis is on the creation of low-molecular regulators of a heterocyclic nature, targeting allosteric sites localized inside the transmembrane tunnel, the most effective of which are TDs. These regulators are endowed with the intrinsic activity of full or inverse agonists and the activity of PAMs and NAMs. They moder-

ately affect LHCGR activity, biasly regulate LH-dependent intracellular cascades, and also retain specific activity when administered orally. Thus, they have a number of advantages over gonadotropin-based drugs, and in the future can be used as prototype drugs for correcting androgen deficiency and in ARTs.

Allosteric sites located in the extracellular and cytoplasmic regions of the receptor can also become targets for the development of allosteric regulators. In this case, peptide regulators corresponding in primary structure to the membrane-proximal regions of ICL2, ICL3 and the C-terminal domain, as well as peptide constructs similar to regions of the hinge region and ECLs of LHCGR, are of particular interest. For ICL-derived peptides with the activity of allosteric regulators of GPCRs, there are very successful developments based on the creation of pepducins (ICL-derived peptides modified by hydrophobic radicals), including for LHCGR, FSHR and TSHR, as well as for the structurally related relaxin receptor [435,437,440,441]. The main obstacle to the widespread use of such peptide constructs is their low resistance to proteolytic degradation, especially considering their enrichment in positively charged AARs. Along with pepducins, allosteric regulators of LHCGR, there may also be LMW compounds that can penetrate the plasma membrane and effectively interact with allosteric sites localized in ICLs and in the intracellular vestibule of the transmembrane tunnel, as shown for parmodulins, intracellular LMW allosteric regulators of protease-activated receptors [428,442].

Finally, it is of particular interest to assess the influence of cholesterol, phospholipids and other membrane lipids on the functional activity of LHCGR through their interaction with allosteric sites located on the lateral surface of the TMD and at its interfaces with extracellular and cytoplasmic loops. In this case, a relationship can be established between the composition of the diet, metabolic disorders and other factors affecting the lipid composition of the membrane, and the activity of LHCGR signaling. It is also impossible to exclude the influence of steroid hormones on the activity of LHCGR, not according to the classical scenario of negative feedback, but through direct interaction with allosteric sites of the receptor.

All this stimulates further research into the allosteric mechanisms of LHCGR functioning and regulation, as well as the search for new technologies to specifically influence the functional activity of the LHCGR signaling system.

Abbreviations

AAR, amino acid residue; AC, adenylyl cyclase; ART, assisted reproductive technology; BAM, biased allosteric modulator; cAMP, 3′-5′-cyclic adenosine monophoshate; CG, chorionic gonadotropin; CREB, cAMP-dependent transcription factor (cAMP Response Element-Binding protein); DAG, diacylglycerol; DM, diabetes mellitus; eCG, equine chorionic gonadotropin; ECL1,2,3, extracellular loops 1, 2 and 3; EPAC1/2, Exchange Protein directly activated by Cyclic AMP, types 1 and 2; ERK1/2, extracellular signal-regulated kinases, types 1 and 2, the effector components of mitogen-activated protein kinase cascade; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; GalNAc, *N*-acetylgalactosamine; G_i, heterotrimeric G proteins that inhibit adenylyl cyclase activity; GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; GPH, glycoprotein hormone; GPHR, glycoprotein hormone receptor; $G_{q/11}$, heterotrimeric G proteins that and stimulate PLCβ and calcium signaling; GRK, G protein-coupled receptor-specific protein kinases; G_s, heterotrimeric G proteins that stimulate adenylyl cyclase activity; hCG, human chorionic gonadotropin; ICL1,2,3, intracellular loops 1, 2 and 3; IP3, inositol-3,4,5-triphosphate; LH, luteinizing hormone; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; LMW, low-molecular-weight; LRR, leucine-rich repeat; MAPKs, mitogen-activated protein kinases; NAM, negative allosteric modulator; PAM, positive allosteric modulator; PDE, cyclic nuleotide-activated phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; PLCβ, phosphoinositide-specific phospholipase Cβ; StAR, cholesterol-transporting protein (Steroidogenic Acute Regulatory protein); TGFβ, transforming growth factor-β; TM, transmembrane helix; TPD, thieno[2,3-d]pyrimidine derivative; TSH, thyroid-stimulating hormone; TSHR, thyroid-stimulating hormone receptor; VEGF, vascular endothelial growth factor.

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Conflict of Interest

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