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Mutations in G protein-coupled receptors that impact receptor trafficking and reproductive function

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Abstract

G protein coupled receptors (GPCRs) are a large superfamily of integral cell surface plasma membrane proteins that play key roles in transducing extracellular signals, including sensory stimuli, hormones, neurotransmitters, or paracrine factors into the intracellular environment through the activation of one or more heterotrimeric G proteins. Structural alterations provoked by mutations or variations in the genes coding for GPCRs may lead to misfolding, altered plasma membrane expression of the receptor protein and frequently to disease. A number of GPCRs regulate reproductive function at different levels; these receptors include the gonadotropin-releasing hormone receptor (GnRHR) and the gonadotropin receptors (follicle-stimulating hormone receptor and luteinizing hormone receptor), which regulate the function of the pituitary-gonadal axis. Loss-of-function mutations in these receptors may lead to hypogonadotropic or hypergonadotropic hypogonadism, which encompass a broad spectrum of clinical phenotypes. In this review we describe mutations that provoke misfolding and failure of these receptors to traffick from the endoplasmic reticulum to the plasma membrane. We also discuss some aspects related to the therapeutic potential of some target-specific drugs that selectively bind to and rescue function of misfolded mutant GnRHR and gonadotropin receptors, and that represent potentially valuable strategies to treat diseases caused by inactivating mutations of these receptors.

Keywords

G protein-coupled receptors; intracellular trafficking; gonadotropin-releasing hormone receptor; luteinizing hormone receptor; follicle-stimulating hormone receptor; pharmacological chaperon; hypogonadism; protein misfolding

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1. Introduction

G-protein coupled receptors (GPCRs) constitute a large superfamily of integral membrane proteins that mediate cellular responses from structurally diverse extracellular signals, including hormones, sensory stimuli, neurotransmitters, proteinases, chemokines, and drugs, by coupling to a repertoire of heterotrimeric G-proteins (Fredriksson et al., 2003; Lagerstrom and Schioth, 2008; Ulloa-Aguirre and Conn, 1998). In turn, activated G proteins regulate specific downstream effectors, such as phospholipases, adenylyl cyclase, protein kinases, and ion channels. Although G protein-coupled receptors vary considerably in molecular size, all share a common molecular topology that consists of a single polypeptide chain which traverses the lipid bilayer seven times, forming characteristic transmembrane (TM) α-helices connected by alternating extracellular and intracellular loops (eL and iL, respectively), with extracellular NH_2-terminus and intracellular COOH-terminal (Ctail) sequences of variable length. This particular type of receptors plays essential roles on a wide range of biological functions (Ulloa-Aguirre and Conn, 2009), and because of their relevance in function and as a cause of disease, they are important therapeutic targets for an array of diseases, as reflected by the fact that ~60–70% of all approved drugs are proposed to selectively target these particular receptors (Lagerstrom and Schioth, 2008; Overington et al., 2006; Schlyer and Horuk, 2006).

The life cycle of GPCRs begins at the endoplasmic reticulum (ER), were synthesis, folding and assembly of proteins occur. Properly folded receptors are then targeted to the ER-Golgi intermediate complex and thereafter to the Golgi apparatus and trans-Golgi network where processing of the protein is completed before delivery to the cell surface plasma membrane (PM); here they become accessible to their cognate ligands (Broadley and Hartl, 2009) (Fig. 1). Interaction between GPCRs and agonists at the PM, provokes endocytosis of the receptor-agonist complex, which is then followed by either recycling of the receptor back to the PM or targeting to the lysosomes and/or proteasomes for degradation. Thus, the balance between trafficking from the ER to the PM and the endocytosis-recycling pathway determines the net amount of receptor protein available to interact with agonist and elicit a measurable biological response. As with any newly synthesized protein, GPCRs are subjected to a strict quality control system (QCS) that monitors and is sensitive to the correctness of nascent protein folding into a three-dimensional structure (Ulloa-Aguirre and Conn, 2009). By monitoring the structural and conformational features of newly synthesized proteins, the QCS determines which proteins must be retained at the ER and eventually degraded or routed to the Golgi apparatus and thereafter to the secretory pathway or appropriate compartment within the cell. Thus, the QCS prevents accumulation of misfolded proteins that may aggregate and interfere with cell function. G protein-coupled receptor export from the ER to the Golgi is modulated by interaction of the proteins with specialized folding factors, escort proteins, retention factors, enzymes, and members of the molecular chaperone families, which belong to the ER QCS and the so-called proteostasis network (Hartl et al., 2011; Hutt et al., 2009; Ron and Walter, 2007). In particular, molecular chaperones are essential components of the ER QCS that evaluate native receptor conformation and promote delivery from the ER to the Golgi where the protein molecule is processed before final delivery of the mature form to the PM (Brooks, 1999; Hartl and Hayer-Hartl, 2002; Morello et al., 2000a). Molecular chaperones are an important quality control mechanism that not only recognizes, but also retains and targets misfolded, non-native protein conformers for their eventual degradation via the polyubiquitination/proteasome pathway (Chevet et al., 2001; Klausner and Sitia, 1990; Schubert et al., 2000; Werner et al., 1996). Some general structural features such as exposure of hydrophobic shapes, unpaired cysteines, immature glycans, and distinct sequence motifs embedded within the receptor sequence are factors important for determining chaperone-protein

association (Angelotti et al., 2010; Broadley and Hartl, 2009; Hartl and Hayer-Hartl, 2009; Schubert et al., 2000; Werner et al., 1996). Some of the effects of molecular chaperones on protein folding may be mimicked by the so-called chemical chaperones (Arakawa et al., 2006; Loo and Clarke, 2007; Robben et al., 2006; Sampedro and Uribe, 2004) and pharmacological chaperones (or pharmacoperones) (Bernier et al., 2004b), which may rescue misfolded receptors *in vitro* and *in vivo*, and thus represent therapeutic options for an array of diseases caused by protein misfolding (Bernier et al., 2006; Conn and Ulloa-Aguirre, 2010; Conn et al., 2007; Ulloa-Aguirre, 2011). Despite their potential application for treating misfolded diseases, there are some drawbacks for the use of chemical chaperones *in vivo*. Effective folding of mutant proteins requires high concentrations of chemical chaperones and although chemical chaperones can rescue some misfolded proteins, they are nonspecific and might in parallel increase secretion or intracellular retention of many different proteins, potentially leading to inappropriate changes in the levels and/or bioactivity, thereby compromising cell function and/or local homeostasis (Bernier et al., 2004b; Castro-Fernandez et al., 2005). In contrast to chemical chaperones, pharmacoperones are target-specific and have the advantage of *selective* association with the misfolded protein, without interfering with the fate of other proteins. Pharmacoperones are small, often hydrophobic, compounds that enter cells and serve as specific molecular templates, promoting correct folding and allowing the target protein to pass the ER QCS and be expressed at the cellular loci where they may function (Arakawa et al., 2006; Bernier et al., 2004b; Conn and Janovick, 2011; Conn and Ulloa-Aguirre, 2011; Loo and Clarke, 2007). Pharmacoperones bind the target protein with high affinity and have been used experimentally in a number of experimental disease models provoked by misfolding and/or abnormal aggregation of particular proteins (Estrada and Soto, 2007, 2006; Hammarstrom et al., 2003; Ishii et al., 2007; Sigurdsson et al., 2000; Soto, 2001), including GPCRs (Bernier et al., 2004a; Bernier et al., 2004b; Bernier et al., 2006; Conn and Ulloa-Aguirre, 2010; Conn et al., 2007).

Defective trafficking of the GPCR from the ER to the PM due to misfolding is now widely recognized as a cause of a large number of loss-of-function diseases, including retinitis pigmentosa, congenital hypothyroidism and obesity, X-linked nephrogenic diabetes insipidus, and reproductive disorders, among others, in which misfolding leads to retention, increased intracellular degradation, and hence, deficient upward trafficking of the misfolded mutant to the PM (Castro-Fernandez et al., 2005; Ulloa-Aguirre and Conn, 2009). Although loss-of-function mutations in GPCRs may modify domains involved in key functions of the receptor (binding to agonist, receptor activation, and interaction with cognate G proteins and/or accessory/scaffold proteins), they also may affect sequences essential for proper folding and traffic of the protein to the PM. Thus, misfolding may give rise to proteins that retain intrinsic function but that are misrouted and thus cannot reach the PM, leading to disease. The fact that some misfolded proteins may retain function, offers the therapeutic opportunity to directly correct misrouting and rescue, partially or completely, function of the mutant receptors, potentially curing disease (Bernier et al., 2006; Conn and Janovick, 2009; Janovick et al., 2002; Loo and Clarke, 2007; Nakamura et al., 2010; Ulloa-Aguirre et al., 2003; Ulloa-Aguirre et al., 2006).

This review focuses on mutations that lead to misfolding and defective trafficking of GPCRs involved in reproductive function in humans. A number of GPCRs regulate reproductive function at different levels; these receptors include the prokineticin-2 receptor (PROKR2), involved in the migration of GnRH-producing neurons from the olfactory bulb to the hypothalamus during embryogenesis; the neurokinin-B receptor and GPR54, which play a pivotal role in the function of the hypothalamic gonadotropin- releasing hormone (GnRH) pulse generator; and the GnRH, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) receptors (GnRHR, FSHR and LHR, respectively), which are directly involved in the

regulation of gonadal function. Although mutations in all these GPCRs may result in misfolding and defective intracellular trafficking of the altered receptor (Francou et al., 2011; Huhtaniemi and Alevizaki, 2007; Martens et al., 2002; Monnier et al., 2009; Nimri et al., 2011; Rannikko et al., 2002; Ulloa-Aguirre et al., 2004), this review focuses on the three latter receptors (the GnRHR, FSHR and LHR), which represent valuable models for the development of pharmacoperone drugs potentially applicable to treat diseases caused by GPCR misrouting.

2. GnRHR trafficking-defective mutants leading to HH

The GnRHR is a GPCR that belongs to family A (rhodopsin/β-adrenergic-like family) of GPCRs (Millar et al., 2004; Ulloa-Aguirre and Conn, 1998). This receptor binds GnRH, which is a decapeptide produced by the hypothalamus and is released to the anterior pituitary where it acts to engender gonadotropin synthesis and secretion (Conn and Crowley, 1994; Ulloa-Aguirre and Timossi, 2000). GnRH signals mainly through activation of the $G_{11/q}$ protein. Besides several unique structural features that distinguish the mammalian type I GnRHR from other GPCRs (Millar et al., 2004), two particular determinants dramatically influence trafficking of the GnRHR: *i.* the lack of a Ctail peptide which projects into the cytosol (Millar et al., 2004) and *ii.* the presence of a lysine residue at position 191 (K^{191}) in the eL2. Other species, such as fish, reptiles, birds, and the primate type II GnRHR contain a Ctail peptide, which plays an important role in determining differential physiological receptor regulation (Blomenrohr et al., 1999; Heding et al., 1998; Lin et al., 1998; McArdle et al., 1999; Millar, 2003). Insertion of the piscine Ctail sequence to the wild-type (Wt) human (h) GnRHR, dramatically increases its PM expression (Janovick et al., 2003b; Maya-Nunez et al., 2011), an effect that has allowed the study of mechanisms that differentially regulate trafficking of this particular receptor (Janovick et al., 2003b; Maya-Nunez et al., 2011; Maya-Nunez et al., 2002). On the other hand, at position K^{191} is frequently found glutamic acid or glycine (ocassionally) in non-primate mammals instead of lysine (Janovick et al., 2006; Ulloa-Aguirre et al., 2006); in fact, in rodents (rat and mice) GnRHRs the orthologous amino acid is absent, thereby yielding a structure that is one residue smaller (327 amino acid residues). The absence of lysine in this position confers the rodent GnRHRs with an increased PM expression (Arora et al., 1999), whereas its presence in the hGnRHR (328 amino acids) *limits* the number of GnRHR molecules that reach the PM after synthesis. The mechanism subserving the effect of K^{191} on PM expression of the primate receptor, includes interfering with formation of the C^{14}-C^{200} disulfide bridge, which is an essential structural requirement to stabilize the hGnRHR in a conformation compatible with ER export (Arora et al., 1999; Janovick et al., 2006; Jardon-Valadez et al., 2009; Maya-Nunez et al., 2002; Ulloa-Aguirre et al., 2006). Several motifs in multiple domains of the hGnRHR control the destabilizing influence of K^{191} on this bridge and limit its PM expression (Janovick et al., 2006; Ulloa-Aguirre et al., 2006); identification of these motifs in the hGnRHR sequence explains why insertion of K^{191} to the rat sequence does not perturb PM expression of the receptor. In fact, in the rat GnRHR the C^{14}-C^{199} bridge is not essential for receptor expression as replacement of either of the cysteine residues of the bridge does not affect PM expression or GnRH-stimulated cellular signaling (Janovick et al., 2006). These data unveiled a novel and underappreciated mechanism for posttranslational control of a GPCR by altering its interaction with the ER QCS and provided a biochemical explanation for the pathogenesis of some disease-causing mutations of this receptor (Conn et al., 2006).

Thirty one inactivating mutations (including deletions of two large sequences) in the hGnRHR have been described as a cause of hypogonadotropic hypogonadism (HH) (Beranova et al., 2001; Conn and Ulloa-Aguirre, 2010; Gianetti et al., 2012; Ulloa-Aguirre et al., 2004), a disease characterized by decreased or apulsatile gonadotropin release and reproductive failure (Gianetti et al., 2012; Ulloa-Aguirre et al., 2004). Eight homozygous

and ~18 heterozygous combinations of hGnRHR mutants have been detected to be expressed by individuals exhibiting either partial or complete forms of HH (Conn and Ulloa-Aguirre, 2010; Gianetti et al., 2012). Patients harboring some of these hGnRHR mutations, also exhibit alterations in other HH-causing genes, such as the *PROKR2* or the fibroblast growth factor receptor 1 gene (Gianetti et al., 2012). Expression of these hGnRHR mutants in *in vitro* heterologous systems resulted in cells that minimally bind GnRH agonists and thus that exhibit no, or marginal response to agonist stimulation by effector activation. Initially, these observations suggested that such mutations provoked structural alterations in motifs involved in agonist binding, receptor activation or interaction with coupled effectors. Nevertheless, the majority (~90%) of the hGnRHR mutants whose function has been examined to date have showed to be related to defects in intracellular trafficking, as revealed by mutational studies and/or response to pharmacologic manipulations (Conn and Ulloa-Aguirre, 2011; Leanos-Miranda et al., 2002; Maya-Nunez et al., 2011). The clinical phenotypes expressed by homozygous or compound heterozygous patients bearing these particular mutations have been summarized previously (Beranova et al., 2001; Gianetti et al., 2012; Karges et al., 2003; Leanos-Miranda et al., 2005; Ulloa-Aguirre et al., 2004).Because reproductive failure is not life-threatening, it is likely that many partial forms of HH go undiagnosed and individual mutants, if severe in phenotype, are not inherited by progeny (Beranova et al., 2001; Gianetti et al., 2012). Such misfolded mutants frequently exhibit gain (*eg*. the $Y^{108}C$ mutant) or loss (*eg*. the $C^{200}Y$ mutant) of either cysteine residues (necessary to form bridges associated with the tertiary structure of proteins), a change in residue charge (*eg*. the $E^{90}K$ hGnRHR mutant, see below), or a proline (an amino acid residue frequently associated with an inflexible turn in the protein backbone fold, *eg*. the $P^{320}L$ mutant). These structural characteristics, as well as motifs involved in the control of the destabilizing influence of K^{191} on the C^{14}-C^{200} bridge formation (see above), explain some of the pathogenic mechanisms whereby mutations in the hGnRHR lead to defective intracellular traffic. For example, in the misfolded $E^{90}K$ mutant (located at the TM2), the dramatic change in amino acid charge blocks the formation of an E^{90}-K^{121} salt bridge required to stabilize the interaction between the TMs 2 and 3, which apparently is a requisite to pass the ER QCS (Janovick et al., 2009b). Furthermore, the native $E^{90}K$ hGnRHR conformation results in a constitutively active receptor as unveiled by pharmacoperone and genetic (*eg*. deletion of K^{191}) interventions, which allowed for plasma membrane localization (Janovick and Conn, 2010). This indicated that the intact TM2-TM3 salt bridge required for correct trafficking is an evolutionary sentinel employed by the ER QCS to protect the cell against PM expression of a constitutively active receptor.

As mentioned above, the amino acid residue K^{191} is important from the evolutionary point of view since the absence of K^{191} confers the rodent GnRHRs with an *increased efficiency* of PM expression, whereas its presence in the human GnRHR limits the number of GnRHR molecules that reach the PM after synthesis (Arora et al., 1999). Consequently, and in contrast to humans, mice homozygous for the $E^{90}K$ mutation, exhibit a much milder phenotype due to partial PM expression of the mutant GnRHR. Whereas male patients harboring this mutation in both alleles exhibit a complete form of hypogonadism, extremely low serum gonadotropin levels, and unresponsiveness to exogenous GnRH stimulation (Soderlund et al., 2001), in male mice harboring the *Gnrhr* $E^{90}K$ mutation both gonadotropins are only modestly reduced in serum and increase after GnRH agonist administration (Stewart et al., 2012). Further, these mice are fertile and only exhibit reduced (albeit histologically normal) testis size. In contrast to males, females homozygous for the mutation are infertile; although they exhibit folliculogenesis, they fail to ovulate and to form corpora lutea, most probably due to reduced sensitivity to endogenous GnRH and inability to decode GnRH pulse frequency and amplitude to produce the gonadotropin response necessary to trigger ovulation (Stewart et al., 2012).

In the case of the $C^{200}Y$ mutant, the substitution prevents formation of the C^{14}-C^{200} bridge required for the hGnRHR to pass the ER QCS (Janovick et al., 2006; Jardon-Valadez et al., 2009; Ulloa-Aguirre et al., 2006). Interestingly, the markedly reduced PM expression of several misfolded mutants ($C^{200}Y$, $C^{14}A$ or $C^{14}S$ mutants) may be completely restored by pharmacoperones and/or by deleting K^{191} (Janovick et al., 2006; Jardon-Valadez et al., 2009). Further, molecular dynamics simulations have shown that when K^{191} is removed from the hGnRHR, the distance between the sulfur or oxygen-sulfur groups of cysteine 14 and 200 is shorter and more stable, and the conformation of both the eL2 and the NH_2-terminal domain become less fluctuating than in the receptor bearing K^{191} (Jardon-Valadez et al., 2009). Contrary to this mutant, the $Y^{108}C$ mutation provokes formation of an aberrant C^{108} (at the eL1)- C^{200} (at the eL2) disulfide bridge, leading to a gross distortion in the conformation of the receptor, preventing its traffic to the PM (Maya-Nunez et al., 2011). It is possible to partially rescue function of this mutant by deletion of K^{191} or pharmacoperone treatment, although complete rescue may be only achieved when pharmacoperone action and deletion of K^{191} are combined (Maya-Nunez et al., 2011). Finally, mutations at positions 168 ($S^{168}K$) and 217 ($S^{217}K$) in the TMs 4 and 5, respectively, are thermodynamically unfavored substitutions that provoke twisting of the corresponding a-helices, moving eL2 away from the NH_2-terminal domain, and preventing formation of the C^{14}-C^{200} bridge. Therefore, these mutant receptors never pass the ER QCS and are completely resistant to genetic or pharmacologic rescue approaches (Janovick et al., 2003a; Janovick et al., 2006; Leanos-Miranda et al., 2002; Ulloa-Aguirre et al., 2006).

It is widely acepted that most GPCRs self associate to form dimers. This proclivity can have detrimental effects in some conditions, such as in autosomal dominant inherited diseases. In such a case, defective PM expression of completely or partially functional receptors might be attributed or amplified by the dominant-negative effect of oligomerization of the defective, misfolded receptor with its Wt counterpart (Conn et al., 2007; Le Gouill et al., 1999; Zhu and Wess, 1998). In the case of the hGnRHR, K^{191} plays an important role in mediating the inhibitory effects of misfolded hGnRHR mutants on Wt receptor PM expression, an effect that is mediated by intracellular retention and aggregation of the mutant-Wt complexes (Brothers et al., 2004). This dominant negative effect might potentially worsen the disease in simple or compound heterozygous patients showing complete or partial forms of HH (Leanos-Miranda et al., 2005; Leanos-Miranda et al., 2003). In this regard, folding of hGnRHR mutants and Wt receptor PM expression may be promoted by pharmacoperones, preventing the dominant negative effect of the former and rescuing Wt receptor function (Leanos-Miranda et al., 2005).

In order to understand the mechanism of action of pharmacoperones on misfolded hGnRHRs, we have recently employed a combined strategy that includes mutagenesis, confocal microscopy, and computer modeling and ligand docking. We have found that a number of different chemical classes of compounds [indoles and quinolones, and the distinctly different erythromycin macrolide, A177775, and TAK-013 (Janovick et al., 2003a; Ulloa-Aguirre, 2011)] stabilize the $E^{90}K$ hGnRHR mutant by promoting formation of a salt bridge associating D^{98} (at the extracellular face of TM1) and K^{121} (at the TM3) (Conn and Janovick, 2009; Janovick et al., 2009b; Jardon-Valadez et al., 2008). This pharmacoperone-mediated bridge functions as a surrogate bond for the highly conserved E^{90}-K^{121} salt bridge (Jardon-Valadez et al., 2008), allowing stabilization of the TM2-TM3 conformation, which is a potential structural requirement for passing the ER QCS and for rescuing function of the hGnRHR to the PM. Since D^{98} and K^{121} are contact points for the natural ligand of the GnRHR (Millar et al., 2004), it is possible that different competitors of GnRH may interact at or near the ligand binding site, which is located in the lateral plane of the PM, a region rich in hydrophobic residues (Jardon-Valadez et al., 2008). The finding that all pharmacoperones tested rescue a number of hGnRHR mutants to different extents (Janovick

et al., 2003a; Leanos-Miranda et al., 2002; Maya-Nunez et al., 2011), despite their wide distribution along the receptor, indicates that in addition to the C^{14}-C^{200} disulfide bridge, the E^{90}-K^{121} salt bridge may be also recognized by the ER QCS. It should be emphasized that the efficiency of pharmacoperones to rescue function of misfolded receptors depends on the structure of the pharmacoperone (that determines selectivity for a given protein), the complexity of misfolding, and the potential alterations in receptor function provoked by the mutation (Morello et al., 2000a; Morello et al., 2000b; Ulloa-Aguirre et al., 2004, 2003). For this reason, functional rescue of misfolded receptors cannot be expected if the mutation affects key domains involved in receptor function.

3. Trafficking-defective mutants of the gonadotropin receptors leading to hypergonadotropic hypogonadism

The FSHR and LHR are the target receptors for the pituitary gonadotropins, which play a key role in reproductive function. These glycoprotein hormone receptors, together with the thyroid-stimulating hormone receptor (TSHR), belong to family A of the GPCR superfamily. FSHR and LHR are expressed by specific cells in the gonads (Vassart et al., 2004), whereas the TSHR is expressed in the thyroid follicular cell. The FSHR is mainly expressed in ovarian granulosa cells where its action is essential for FSH induced maturation of ovarian follicles and granulosa cell progesterone and estrogen production; in the testis, the FSHR is expressed by the Sertoli cells in the seminiferous tubules, where it supports Sertoli cell growth and metabolism, initiating spermatogenesis. In males, the LHR is expressed mainly in the testicular Leydig cells nests between the seminiferous tubules, where LH induces testosterone production. In females, LHR is expressed in the ovarian theca cells which line the developing follicle and where LH induces androgen production that serves as a substrate for FSH induced P_{450} aromatase, the enzyme that converts androgen to estrogen. LH also is responsible for the follicular breakage and the subsequent release of the oocyte (ovulation).

In contrast to the GnRHR, glycoprotein hormone receptors exhibit a large NH_2-terminal extracellular domain (ECD) that bind their cognate ligands (Ascoli et al., 2002; Dias et al., 2002; Simoni et al., 1997; Ulloa-Aguirre and Conn, 1998). Amino acid sequences of the extracelllar domain of the gonadotropin receptors are 46% identical; they exhibit a NH_2-terminal cysteine-rich region, a central region bearing a structural motif of nine imperfect leucine-rich repeats (LRRs) that is shared with a number of other membrane receptors involved in ligand selectivity and specific protein-protein interactions (Bogerd, 2007), and a carboxyl-terminal cysteine-rich sequence, the so-called hinge region, which structurally links the leucine-rich ECD with the serpentine transmembrane domain of glycoprotein hormone receptors (Fan and Hendrickson, 2005; Jiang et al., 2012). Depending on the glycoprotein hormone receptor, this latter region may be involved in high-affinity hormone binding, receptor activation, intramolecular signal transduction, and/or silencing the basal activity of the receptor in the absence of ligand (Mueller et al.). The mature human hFSHR protein consists of 678 amino acid residues long, whereas the processed LHR is 675 amino acids long (Dias et al., 2002).

Upon agonist binding, the activated FSH and LH receptors stimulate a number of intracellular signaling pathways. The canonical Gαs/cAMP/PKA signaling pathway has been recognized as a key effector mechanism of LH and FSH biological action for more than 20 years (Hunzicker-Dunn and Maizels, 2006). However, gonadotropin receptors have also been reported to couple to other G protein subtypes and activate a number of distinct effector enzymes [reviewed in (Ulloa-Aguirre et al., 2011)], depending on the particular developmental stage of the host cells (Musnier et al., 2009).

Similar to other GPCRs, the gonadotropin receptors have to be correctly folded into a conformation to pass the QCS and be compatible with ER export (Ulloa-Aguirre and Conn, 2009). In fact, mutations that affect the folding process of these receptors result in intracellular retention of the folding intermediates, and eventually in disease. Several GPCR interacting proteins that support folding and trafficking to the PM have been identified to interact with the gonadotropin receptors during their residency at the ER. Coimmunoprecipitation experiments have shown that the folding process of the FSHR and LHR precursors involves interactions with the chaperones calnexin and calreticulin, which bind a broad range of glycoproteins facilitating proper folding of intermediate molecules (Mizrachi and Segaloff, 2004; Rozell et al., 1998), as well as with the protein disulfide isomerase PDI (Mizrachi and Segaloff, 2004), which is an ER-resident enzyme involved in disulfide bond formation of folding intermediates, and that probably acts as a co-chaperone with calnexin and calreticulin during their association with the glycoprotein hormone receptors. No interaction of these receptors with the chaperones BiP (a chaperone that maintains proteins in a state competent for subsequent folding and oligomerization, and that mediates retrograde translocation of misfolded conformers for proteosomal degradation), Grp94 (involved in folding and assembly of membrane proteins of the secretory pathway) or Erp57 (a thiol-disulfide oxidoreductase that catalyzes disulfide bond formation and isomerization, and which is thought to be a cochaperone that physically associates with calnexin and calreticulin in the nascent polypeptide chain) (Gething, 1999; Ulloa-Aguirre and Conn, 2009; Weekes et al., 2012), were detected in these studies. Studying the interaction of gonadotropin receptor mutants with these chaperones has been important to detect differences in folding among mutants (see below).

Properly folded and assembled secretory proteins are segregatd from ER-resident proteins into COPII-coated vesicles for exporting to the Golgi to be further processed before being sent to their final destination. Several recently identified motifs have been shown to be involved in GPCR exit from the ER and the Golgi. Among these motifs is the $F(X)_6LL$ sequence identified in the Ctail of several GPCRs, including the gonadotropin receptors (Duvernay et al., 2004). In the hFSHR, this export motif is located between amino acid residues 633 and 641, whereas in the hLHR this motif is located between residues 630 and 638 (Ascoli et al., 2002; Dias et al., 2002; Ulloa-Aguirre and Timossi, 1998). The Ctail peptide of the hFSHR contains the minimal BBXXB motif reversed in its juxtamembrane region (residues 631 to 635) (Timossi et al., 2004), which in other GPCRs is involved in G protein coupling (Okamoto and Nishimoto, 1992). The last two residues of this motif (R^{634} and R^{635}) and the preceding F^{633} constitute the NH_2-end of the highly conserved $F(X)_6LL$ motif, and thus it was not surprising that mutations in these residues impaired receptor trafficking and cell surface PM localization of the receptor (Timossi et al., 2004; Zarinan et al., 2010). Further, some of these laboratory-manufactured mutant hFSHRs (eg. $R^{635}A$) exert potent dominant negative effects on Wt receptor PM expression (Zarinan et al., 2010), as it has also been observed for some naturally occurring hLHR mutants (Tao et al., 2004). The iL3 of the hFSH and hLH receptors also contains this BXXBB motif (residues 569 to 573) and either deletion or replacement of the basic residues of this motif with alanine impaired PM expression of the modified receptors (Schulz et al., 1999; Timossi et al., 2004). Human FSHRs with point mutations in this sequence (eg. hFSHR R573A) are retained intracellularly, and when co-transected with the Wt receptor also exert strong dominant negative effects, probably by forming misfolded mutant:wild-type receptor complexes (Zarinan et al., 2010) (see below). Another motif identified in all glycoprotein receptors is the AFNGT motif (amino acid residues 193 to 197 in the hLHR and 189 to 193 in the hFSHR), which bears a potential glycosylation site ($N^{195}GT$ and $N^{191}GT$, in the human LH and FSH receptors, respectively). Although mutations in this motif might interfere with the integrity of the LRRs and thus with binding of agonists, it has been shown that mutations in this region rather influence receptor folding and trafficking to the PM (Gromoll et al., 2002;

Rannikko et al., 2002)(see below). In fact, mutations in the first three amino acid residues of this motif lead to altered agonist-stimulated second messenger production without grossly affecting ligand binding affinity (Gromoll et al., 2002). Further mutation of the putative glycosylation site present in this motif (N195I in the hLHR and N^{191}I in the hFSHR) resulted in reduced cAMP production by both receptors, but was more prominent for the hFSHR (Gromoll et al., 2002), thus emphasizing on the importance of glycosylation at this particular residue in the latter receptor.

Two posttranslational modifications are particularly important for intracellular trafficking of the gonadotropin receptors: glycosylation and palmitoylation. Glycosylation occurs during biosynthesis and facilitates folding of protein precursors by increasing their solubility and stabilizing protein conformation (Helenius and Aebi, 2004). The ECD of the hFSHR bears four potential glycosylation sites (as defined by the consensus sequence N-*X*-Ser/Thr, where *X* is any amino acid except proline) at positions 191, 199, 293, and 318 (Dias et al., 2002), whereas the hLHR exhibits six glycosylation sites (at positions 99, 174, 195, 291, 299, and 313). The only direct biochemical evidence that exists as to which sites are glycosylated in the hFSHR comes from the crystal structure of the ECD residues 25–250 (Fan et al., 2005; Jiang et al., 2012). The structures show that carbohydrate is attached at residue N^{191} which protrudes into solvent, whereas no carbohydrate is attached at residue N^{199} which protrudes from the flat beta sheet into the hormone-receptor binding interface. No structural information is available for residues 293 and 318 at this time. Studies with the rat FSHR have suggested that the FSHR is glycosylated at two of three glycosylation consensus sequences (191, 199, and 293) and that the presence of carbohydrates at either one of these residues (N^{191} or N^{293}) is sufficient for receptor folding and trafficking to the PM (Davis et al., 1995). In contrast, abrogation of glycosylation of the rat LHR, did not modify agonist binding, suggesting that the deglycosylated receptor folded properly and trafficked to the PM. These studies underline the differential effects of glycans on the LH and FSH receptor with respect to folding and PM expression.

Another post-translational modification important for GPCR trafficking to the PM is palmitoylation. Cysteine residues in the COOH-terminus of several GPCR have been shown to be the target for S-acylation with palmitic acid; in some receptors this posttranslational modification is often required for efficient delivery of the protein to the cell membrane (Blanpain et al., 2001; Fukushima et al., 2001; Percherancier et al., 2001; Resh, 2006; Tanaka et al., 1998). The hFSHR exhibits in its Ctail two conserved cysteine residues (at positions 646 and 672) and one non-conserved cysteine residue at position 644. Although the hFSHR is palmitoylated at all cysteine residues, regardless of their location in the Ctail of the receptor (Uribe et al., 2008), S-acylation at C^{627} and C^{655} is not essential for efficient FSHR cell surface membrane expression, whereas at C^{629} it is, as replacement of this residue with glycine or alanine reduced detection of the mature form of the receptor by ~40–70%. Further, when all palmitoylation sites are removed from the FSHR, cell surface PM expression is reduced to ~10–30% of that shown by the Wt receptor (Uribe et al., 2008). The hLHR is palmitoylated at two conserved cysteine residues (643 and 644) (Kawate and Menon, 1994) but in contrast to the hFSHR, palmitoylation of this receptor is not important for trafficking to the PM, as abrogation of palmitoylation did not appear to affect PM expression and agonist binding (Kawate and Menon, 1994; Munshi et al., 2005).

As with many other GPCRs, gonadotropin receptors constitutively form dimers or oligomers during receptor biogenesis (Guan et al., 2009; Tao et al., 2004; Thomas et al., 2007), which may impact on the intracellular trafficking of the receptor to the PM as well as for signal diversification through coupling to multiple G proteins (Breitwieser, 2004; Marshall et al., 1999; Nechamen et al., 2007; Szidonya et al., 2008). Although the mechanisms and extents of the hLHR and hFSHR self-association are still incompletely known, studies on these and

other GPCRs suggest that association between receptors might occur at multiple contact sites, including the ECD and the TM regions (Fan and Hendrickson, 2007, 2005; Fan et al., 2005; Guan et al., 2010; Hebert et al., 1996; Jiang et al., 2012; Lee et al., 2003; Ng et al., 1996; Zarinan et al., 2010). As discussed above for the hGnRHR, misfolded hFSH and hLH receptors also associate with their immature Wt counterparts, and prevent Wt receptor traffickng to the PM (Tao et al., 2004; Zarinan et al., 2010). In fact, cotransfection of the $A^{593}P$ or $S^{616}Y$ misfolded hLHR mutants with the Wt receptor in HEK-293 cells, resulted in a 40–50% reduction in cell surface PM expression as compared to cells transfected only with the Wt receptor (Tao et al., 2004). Likewise, cotransfection of the the Wt receptor with increasing amounts of the laboratory-manufactured $R^{573}A$ (at the iL3) or $R^{635}A$ (at the Ctail) hFSHR mutants led to a dose dependent reduction in both ligand binding and signal transduction. Interestingly, the dominant negative effect of the mutants was prevented when the transfection mix included also Wt hFSHR fragments involving transmembrane domains or the transmembrane domain 7 and/or the Ctail peptide (Fig. 2)(Zarinan et al., 2010), strongly suggesting that these regions were involved in the intracellular mutant-Wt receptor association. The fact that individuals who are heterozygous for misfolded mutations in the gonadotropin receptors do not exhibit detectable reproductive abnormalities, suggest that the decrease in PM expression of the Wt receptor that results from the dominant negative effect of the misfolded mutant is not low enough to impact on cell function, given that occupancy of only a low fraction of gonadotropin receptors per cell is sufficient to elicit normal responses (Huhtaniemi et al., 1982). In this regard, it is interesting that heterozygous subjects bearing misfolded TSHR mutants (*eg.* $C^{41}S$, $L^{467}P$, and $C^{600}R$) expressed a clinical phenotype of partial TSH resistance presumably due to the dominant negative effect of the mutant receptors on Wt receptor PM expression (Calebiro et al., 2005), underlining the potential effects of misfolded receptors on the dominant transmission of partial GPCR resistance in heterozygous individuals.

Loss-of-function mutations in the gonadotropin receptor genes may lead to disease, whenever both alleles are affected by a mutation, as it occurs in individuals who are homozygous or compound heterozygous for mutations in the hFSHR or hLHR genes. Several naturally occurring inactivating mutations (which include point mutations, amino acid insertions or deletions, or premature truncations) scattered throughout the polypeptide chain of the hLH and hFSH receptor molecules have been described. In 46, XY individuals, loss-of-function mutations in the LHR gene leads to an array of phenotypes, from severe genital ambigüity [due to unresponsiveness of the fetal Leydig cell to CG stimulation –the so-call Type 1 Leydig cell hypoplasia (LCH)-] to cryptorchidism and micropenis (Type II LCH), depending on the severity of the functional deficit provoked by the mutation (Table 1). Women with inactivating mutations in the hLHR display pubertal development, but frequently exhibit primary or secondary amenorrhea and infertility. Meanwhile, inactivating mutations of the hFSHR in men lead to impaired quality of spermatogenesis with normal androgen production, which probably contributes to fertility preservation (Tapanainen et al., 1997). In women bearing inactivating FSHR mutations, the panorama is completely different and comprises an array of phenotypes ranging from lack of pubertal development and primary amenorrhea, with arrest of follicular maturation between primordial and preantral stage and complete resistance to FSH stimulation, to secondary amenorrhea and premature ovarian failure (Aittomaki et al., 1995; Huhtaniemi and Alevizaki, 2007) (Table 2). In either case, the level of residual, functional receptors at the PM, has been shown to correlate with the severity of the clinical phenotype expressed by the patients bearing inactivating mutations in these receptors (Huhtaniemi and Alevizaki, 2007; Huhtaniemi and Themmen, 2005; Touraine et al., 1999), which is an important determinant for the response to exogenous gonadotropins (Vaskivuo et al., 2002).

Among the 24 hLHR mutants described so far, at least nine are trafficking defective receptors in which the net amount of functional receptors expressed at the PM is decreased to a variable extent. These trafficking defective/misfolded receptors bear mutations either in their ECD [$V^{144}F$, $F^{194}V$, $C^{343}S$, and a 33-bp insertion between amino acid residues 18 and 19, immediately upstream of the signal peptide cleavage site (Gromoll et al., 2002; Martens et al., 1998; Richter-Unruh et al., 2002; Richter-Unruh et al., 2004; Wu et al., 1998)] or the TMs 5–7 [$C^{543}R$, $A^{593}P$, delL^{608}-V^{609}, $S^{616}Y$, and $I^{625}K$ (Kremer et al., 1995; Latronico et al., 1996; Latronico et al., 1998; Laue et al., 1996; Martens et al., 2002; Martens et al., 1998; Richter-Unruh et al., 2002; Toledo et al., 1996)]. Some of these mutant hLHRs are interesting from the structural and functional points of view. The $F^{194}V$ misfolded mutant bears the substitution in a highly conserved motif of the gonadotropin receptors (^{193}AFNGT^{197} at the LHR ECD) that contains the N^{195}GT glycosylation motif. This mutation severely impairs trafficking of the mutant receptor to the PM without significantly altering agonist affinity (Gromoll et al., 2002), and leads to LCH. In the case of the inactivating $A^{593}P$ and $S^{616}Y$ mutant hLHRs, which provoke severe or moderate forms of LCH, respectively, both exhibited normal ligand binding affinity but the response to agonist was absent or severely impaired due to misfolding and intracellular retention of the mutant receptors (Martens et al., 2002; Mizrachi and Segaloff, 2004). Further, it was shown that these particular mutants are conformationally distinct and display different folding conformations during their maturation at the ER as suggested by their differential association with molecular chaperones in co-immunoprecipitation studies (Mizrachi and Segaloff, 2004). Whereas similar to the Wt hLHR, the $A^{593}P$ and $S^{616}Y$ mutants were found associated with calnexin, only $S^{616}Y$ was coimmunoprecipitated with PDI; further, although both $A^{593}P$ and $S^{616}Y$ mutants appeared to interact with BiP only the former was found to interact with Grp94 (Mizrachi and Segaloff, 2004). Some mutant hLHRs exhibiting further defects, in addition to misfolding, have also been described. This is the case of the delLeu^{608}/Val^{609} misfolded mutant, which in spite of being expressed at low levels at the PM and to exhibit normal binding affinity, it is unable to activate the G_s protein upon exposure to agonist (Latronico et al., 1998). The same appears to occur with the $I^{625}K$ mutant, which leads to partial LCH; *in vitro* studies showed that in addition to low PM expression, the mutant exhibited decreased coupling efficiency. Since in addition to provoke defective folding and intracellular retention of the protein, the mutations also affect an intrinsic function of the receptor (*eg.* signal transduction), it might be expected that the benefit of pharmacoperone treatment to correct function will be limited (see below).

In contrast to the LHR, which seems insensitive to effects on hormone binding when trafficking is impaired by mutagenesis, the FSHR is particularly sensitive to mutations of the primary sequence. When mutations impair trafficking of this receptor from the ER to the PM (Rozell, 1995; Song et al., 2001), hormone binding activity is severely impaired (Nechamen and Dias, 2003; Rozell, 1995; Song et al., 2001), which may be due to differences in stability between these receptors. In fact, naturally occuring mutations in the ECD of this receptor (*eg.* $I^{160}T$, $A^{189}V$, $N^{191}I$, $D^{224}V$), drastically impair targeting of the receptor to the PM, and thereby limit hormone binding and agonist-stimulated intracellular signaling. Meanwhile, the majority of mutations at or near the transmembrane region (*eg.* $A^{419}T$, $R^{573}C$, $L^{601}V$) have modest effects on FSH binding capacity but impair to variable extent signal transduction, suggesting that, in general, the location of the mutation is a strong determinant of the functional response.

Loss-of-function mutations of the hFSHR are less frequent than those in the hLHR. In fact, only 12 inactivating mutations in the hFSHR receptor have been reported to date (Achrekar et al., 2010; Aittomaki et al., 1995; Allen et al., 2003; Beau et al., 1998; Gromoll et al., 1996a; Kotlar et al., 1997; Meduri et al., 2003; Nakamura et al., 2008; Tapanainen et al., 1997; Touraine et al., 1999). As it has been observed for the inactivating hLHR mutants,

there is in general a good correlation between the residual activity exhibited by the mutant hFSHRs and the severity of the clinical phenotype expressed by the patients bearing the mutation(s) (Aittomaki et al., 1996; Huhtaniemi and Alevizaki, 2007; Huhtaniemi and Themmen, 2005). The exception is the $F^{591}S$ mutation for which no clinical phenotype has been described; this particular mutation, which leads to severely impaired agonist binding and intracellular signaling, was found to be associated with the development of sex cord tumors (Kotlar et al., 1997). Among the 12 mutant hFSHRs reported to date, at least five are trafficking defective receptors which have been detected as intracellular retained molecules [$I^{160}T$, $A^{189}V$, $N^{191}I$, $D^{224}V$, at the ECD (Beau et al., 1998; Gromoll et al., 2002; Gromoll et al., 1996b; Touraine et al., 1999); and $P^{519}T$ at the eL2 (Meduri et al., *2003*)] by *in vitro* studies. The functional defects of the $V^{221}G$ (at the ECD) (Nakamura et al., 2008) and $A^{575}V$ (at the TM6) (Achrekar et al., 2010) mutant hFSHRs have not been studied in detail, whereas in the case of the $P^{348}R$ hFSHR (located at the hinge region of the receptor), both ligand binding and agonist-stimulated signaling were severely impaired (Allen et al., 2003). Whether these three hFSHR mutations interfere with proper trafficking of the receptor from the ER to the PM is still unknown.

The naturally occurring mutation $A^{189}V$ cause a profound defect in targeting of the receptor protein to the cell membrane (Huhtaniemi and Themmen, 2005; Rannikko et al., 2002), confirming the importance of the AFNGT motif for intracellular trafficking of the receptor. As described above, A^{189} is the first amino acid in the perfectly conserv+ed stretch of five amino acids in the gonadotropin receptors (^{189}AFNGT^{193} in the hFSHR), which is also the locus of loss-of-function mutations in the hLHR (Gromoll et al., 2002) (see above). Valine in this position as well as isoleucine in position 191 may interfere with structural integrity of the LRRs which hosts the glycosylation site, and perturbation of this structure likely impairs proper receptor LRR formation, particularly its a-helical portion. Although the putative loss of glycosylation may affect folding and trafficking of the mutant receptor to the PM, it has yet to be determined whether this form of the receptor is glycosylated or not at the N^{191} site. When the $A^{189}V$ mutant is overexpressed *in vitro*, only a very small proportion of the mutated receptor is present at the PM but is functional (Rannikko et al., 2002; Tranchant et al., 2011). Instead, most of the mutated receptor is sequestered inside the cell, explaining the inactivation mechanism (Rannikko et al., 2002). Interestingly, the reduced level of PM expression of the $A^{189}V$ hFSHR confers preferential coupling to the β-arrestin-mediated signaling pathway, similar to that observed when the Wt receptor is expressed at low PM densities, indicating that the selective signaling is due to the low level of PM expression rather than to the mutation itself (Tranchant et al., 2011). This observation might help to clarify why mutations of FSHβ are more deleterious to male fertility than the hFSHR $A^{189}V$ mutation (Layman et al., 2002; Lindstedt et al., 1998; Tapanainen et al., 1997), which preserves a fraction of its receptor signaling repertoire.

The case of the $N^{191}I$ mutation is also interesting. This mutation was originally detected in the heterozygous state in a phenotypically normal women (Gromoll et al., 1996b). Although the mutation affects the putative glycosylation site at position 191 of the receptor, curiously no studies on the effect of this mutation on the binding capacity and PM expression of the FSHR have been performed. Further, *in vitro* studies have been somehow contradictory regarding the impact of the $N^{191}I$ mutation on agonist-stimulated signaling: in one study, FSH-stimulated cAMP production was severely impaired (Gromoll et al., 1996b), whereas in a later study performed by the same group, it was found only modestly decreased (by ~40% relative to that exhibited by the Wt receptor) (Gromoll et al., 2002), suggesting that a fraction of the transfected receptor was, in fact, present at the PM. Given that glycosylation in only one position at the ECD is sufficient for folding and trafficking of the FSHR to the PM (Davis et al., 1995), these studies suggest that the limited PM expression of the mutant

receptor is mainly due to the alteration in the structural integrity of the AFNGT motif, rather than to the absence of glycosylation at this particular site.

Another interesting hFSHR mutant is $R^{573}C$; this mutation does not affect PM expression of the receptor but partially impairs agonist-stimulated signaling (Beau et al., 1998). Nevertheless, when R^{573} is replaced with alanine (Zarinan et al., 2010), the PM expression of the mutant receptor is severely impaired, thus indicating that the location of the mutation and the nature of the amino acid substitution are both strong determinants of the functional features exhibited by mutant hFSHRs. In fact, the $P^{519}T$ mutation in the center of the eL2, leads to complete failure to bind agonist and trigger intracellular signaling; women homozygous for this mutation express a severe phenotype including absence of puberty, primary amenorrhea and small ovaries (Meduri et al., 2003). The effects of this particular mutation contrast with those provoked by other mutations in the serpentine region of the hFSHR ($A^{419}T$, $R^{573}C$, and $L^{601}V$), which usually result in partial receptor inactivation, with minimal effects on FSH binding (Beau et al., 1998; Doherty et al., 2002; Touraine et al., 1999). Therefore, it seems that the loss of a proline at position 519 provokes a severe conformational defect that leads to trapping of the receptor at the ER (Meduri et al., 2003). Because the peptide backbone of proline is constrained in a ring structure, occurrence of this amino acid is associated with a forced turn in the protein sequence, which is likely lost by the substitution with the more reactive threonine; it is thus possible that the abrupt turn at the middle of the eL2 is probably a requisite not only for activity (Dupakuntla et al., 2012) but also for routing.

As mentioned above, the success of pharmacological treatment of misfolded GPCRs with pharmacoperones depends on several factors, including the partial or complete integrity of domains involved in ligand binding, receptor activation and/or coupling to effectors. As demonstrated in the hGnRHR mutants, misfolded hFSH and hLH receptors may also be rescued *in vitro* by pharmacoperone treatment that correct folding and promote trafficking of the intracellularly trapped receptors from the ER to the cell surface PM. In the case of the misfolded A189V hFSHR mutant (which is the prototype of a misfolded hFSHR due to a point mutation), we tested the effect of Org41841, which is a thienopyr(im)idine molecule reported to bind a conserved region of the hLHR without competing for the LH binding site and the site for interaction with effector (*ie*. an allosteric modulator)(van Straten et al., 2002). Exposure of COS-7 cells transfected with the $A^{189}V$ hFSHR expression plasmid to Org41841 increased almost by two-fold PM expression and FSH-stimulated cAMP production of the $A^{189}V$ mutant without significantly altering mARN expression of the receptor nor its ligand binding affinity (Janovick et al., 2009a) (Fig. 3A). Shortly after this report, Newton and colleagues (Newton et al., 2011) found that another cell-permeant, allosterically binding small molecule agonist (Org 42599) rescued folding, PM expression and function of two hLHR misfolded mutants ($A^{593}P$ and $S^{616}Y$) that lead to LCH (Fig. 3B). Major challenges include determining the molecular mechanisms by which these allosteric compounds stabilize and rescue these particular misfolded receptors, and to identify new, highly specific molecules that do not interfere with agonist binding or activation of the misfolded receptor and that may function well *in vivo*. Meanwhile, these studies indicate that treatment of patients bearing mutations that lead to misfolding of gonadotropin receptors may be feasible, particularly in those cases with mild clinical phenotypes.

4. Conclusions

Intracellular retention of misfolded GPCRs is a common cause of human disease. In fact, more than 30 disorders are associated with mutations in GPCRs, which make these proteins a target for drug development (Lagerstrom and Schioth, 2008; Overington et al., 2006;

Schlyer and Horuk, 2006). Although the majority of pharmacoperones identified that rescue misfolded GPCRs are mainly antagonists of the natural ligand detected from "hits" in screens designed to detect antagonists or agonists, new, fully automated high-throughput assays for identification of "pure" pharmacoperone drugs of mutant GPCRs have been recently described (Smithson et al., 2013). These approaches allow identification of structures *missed* in screens designed to select only agonists or antagonists, and thus offer the advantage of identifying new classes of drugs which are specific and rescue active, but do not have inherent modulatory activity (stimulatory or inhibitory). Elucidation of the crystal structure of the GPCRs discussed in this article, will undoubtedly facilitate the design of novel small molecules that may rescue misfolded receptors by specifically targeting those motifs and conformations that influence directly receptor trafficking and that may be altered by mutations in the protein molecule. Pharmacoperone compounds currently constitute the most promising therapeutic approach for treating diseases caused by misfolded GPCRs. Given that ample evidence exists to demonstrate that certain mutations found in GPCRs which bind ligands that are essential for reproductive function cause dysfunction due to aberrant trafficking of otherwise functional proteins, the mutations discussed above provide the vehicle to identify and assess such kind of drugs.

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Highlights

- GPCRs are subjected to conformational scrutiny at the endoplasmic reticulum prior to processing and trafficking to the cell surface membrane.
- Point mutations in genes coding the gonadotropin-releasing hormone, the luteinizing hormone and the follicle-stimulating hormone receptors, may result in misfolding and intracellular trapping of the abnormal receptor protein.
- Mutations in the GnRHR and gonadotropin receptors lead to hypo- or hypergonadotropic hypogonadism in humans.
- Pharmacoperones are a useful strategy to rescue function of mutant GPCRs, including the GnRHR and the gonadotropin receptors.

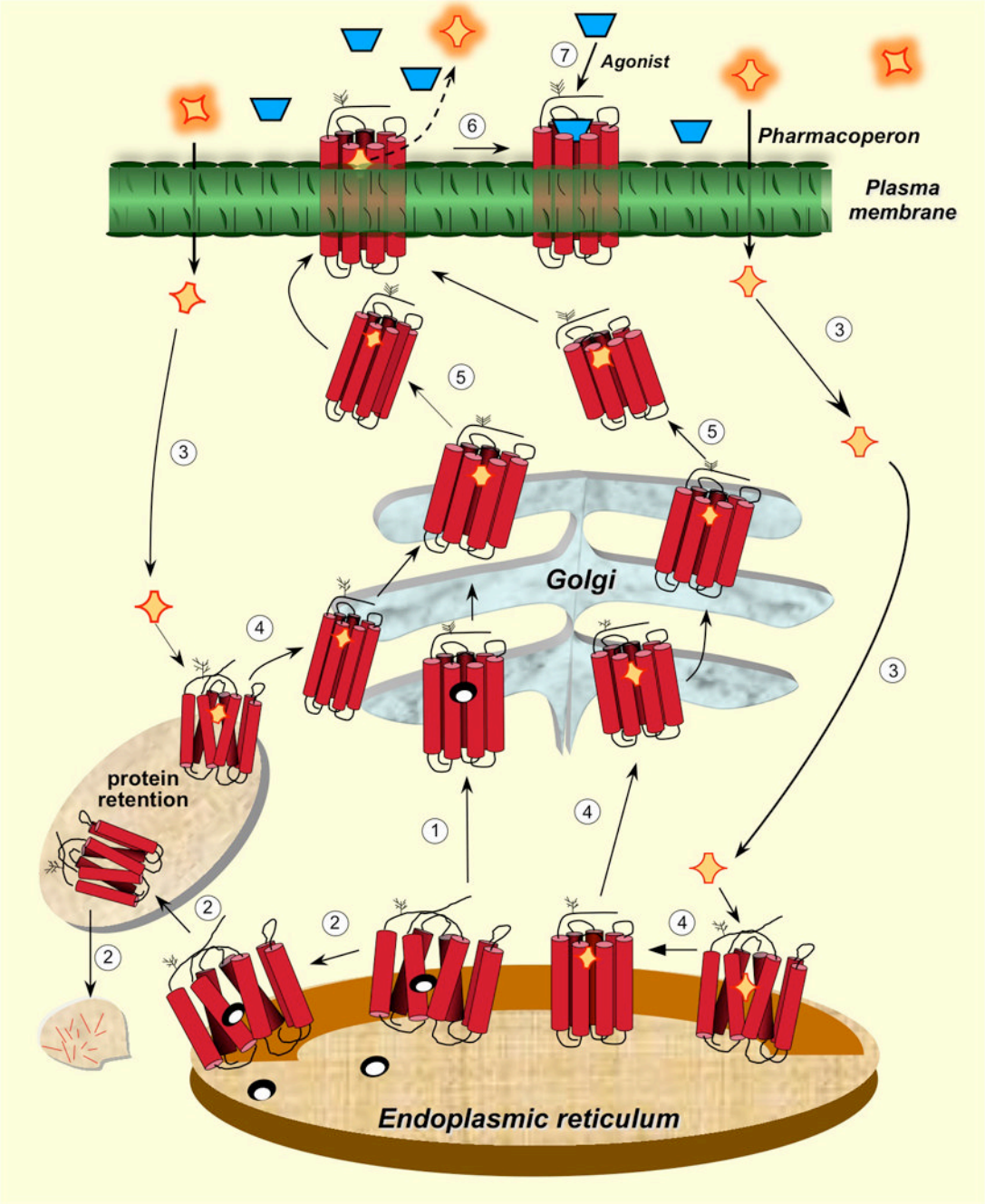

Figure 1.

Intracellular trafficking of GPCRs from the ER to the plasma membrane and relation to the cellular quality control system. Newly synthesized proteins are translocated to the lumen of the ER where folding is both facilitated and/or corrected by molecular chaperones (oval structures). The correctly folded protein is thereafter exported to the Golgi complex for further processing (*eg.* glycosylation) (step 1 in clear circles) and routing to the plasma membrane. When folding fails, misfolded proteins are retained in the ER and eventually targeted for degradation through the polyubiquitination/proteasome pathway (step 2). Pharmacoperones (orange rhomboid structures) diffuse into the cell (step 3) and selectively bind the misfolded protein to correct misfolding promoting its export to the Golgi complex

(step 4). Previously synthesized misfolded proteins, retained by the QCS, may still be rescued by pharmacoperones (steps 3 and 4 at the left). Mature processed proteins are then delivered to the cell surface PM (step 5), where the pharmacoperone can dissociate from the target protein (step 6) allowing the receptor to interact with agonist (step 7). Development of pharmacoperones that dissociate functions or rescue from agonism is currently needed as many pharmacoperone drugs are also receptor agonists/antagonists. Modified from Conn and Ulloa-Aguirre (2011), with permission of the publisher.

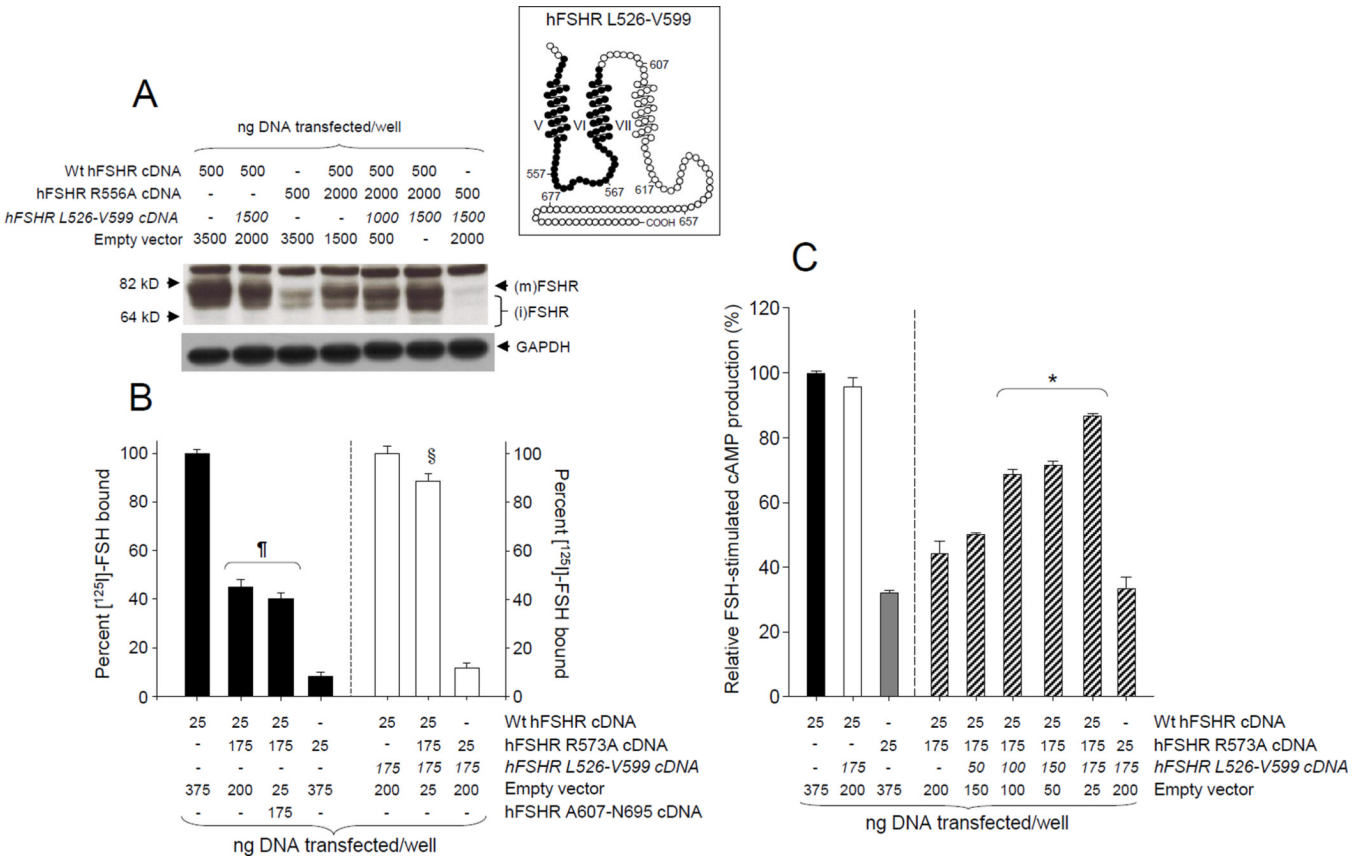

Figure 2.

Dominant negative effect of the laboratory-manufactured R573A mutant hFSHR on Wt hFSHR PM expression (**A** and **B**) and function (**C**), and effect of co-transfecting the Wt hFSHR L526-V599 fragment (*inset*, black circles) cDNA on the dominant negative effects of the mutant. (**A**) Representative autoradiogram from an immunoblot of the hFSHR present in protein extracts from HEK-293 cells transfected with the cDNAs shown at the top of the blot. The autoradiograph was overexposed in order to show the expression levels of the mutant receptor species and the immature forms of the hFSHR. (**B**) HEK-293 cells were co-transfected with the cDNAs shown at the bottom of the graph and specific [^{125}I]-FSH binding was determined in the presence or absence of the L526-V599 fragment. Note that in contrast to the L526-V599 fragment, co-transfection with the hFSHR A607-N695 cDNA fragment (encoding the TM7 and the entire Ctail of the Wt receptor), did not affect expression of the Wt receptor co-transfected with the R573A mutant. (**C**) Maximal FSH-stimulated total cAMP accumulation in HEK-293 cells co-transfected with the cDNAs shown at the bottom of the graph; note that the L526-V599 fragment cDNA was co-transfected in increasing concentrations. The Wt and mutant hFSHR cDNAs were hosted by the pSG5 vector whereas the hFSHR fragments were in pcDNA3.1. The results shown in **B** and **C** are the mean ± SEM from three independent experiments. *$p < 0.05$ *vs* all other conditions; $^{¶}p < 0.01$ *vs* all other conditions; $^{§}p < 0.05$ *vs* Wt + L526-V599 fragment + empty vector. (m)FSHR, mature form of the hFSHR; (i)FSHR, immature form of the hFSHR. Co-transfection of the mutant and Wt hFSHRs resulted in decreased PM expression and function of the mature form of the hFSHR as well as reduced specific [^{125}I]-FSH binding. Co-transfection of the Wt and mutant FSHRs with the L526-V599 fragment led to almost complete functional recovery and PM expression of the hFSHR. Reproduced from Zariñán et al., (2010), with permission of Elsevier Ireland LTD.

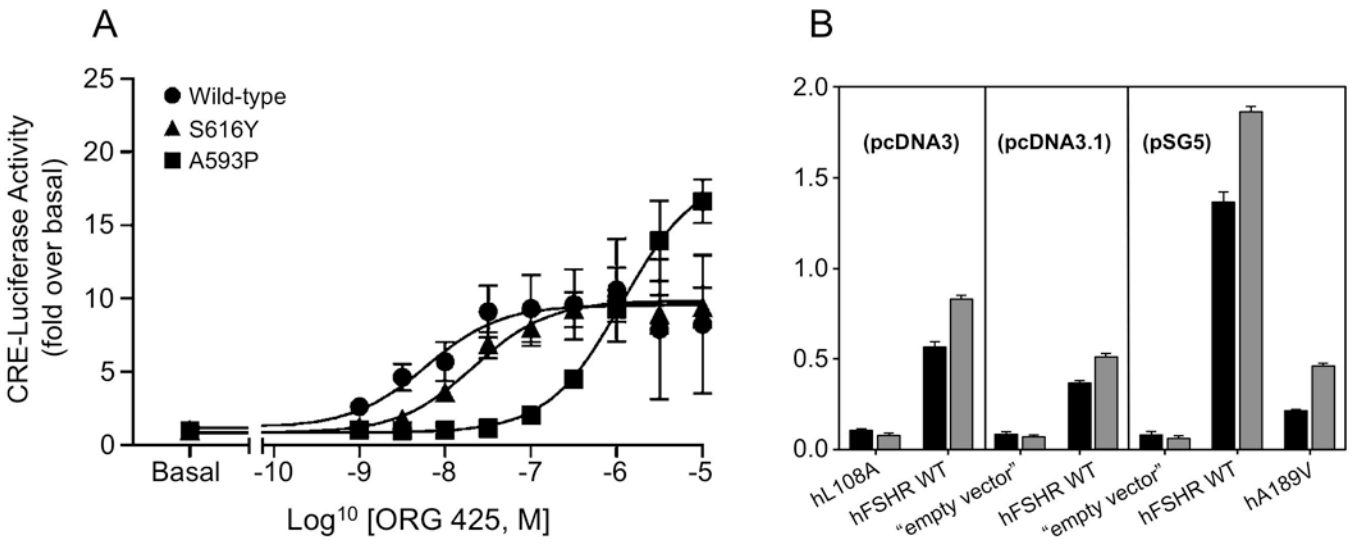

Figure 3.

Rescue of hLHR and hFSHR misfolded mutants by pharmacoperones. (**A**) Measurement of cAMP accumulation by CRE-luciferase reporter gene activaton after 24 h stimulation in cells expressing Wt (●), A593P mutant (■), or S616Y (▲) mutant hLHRs was determined over a range of concentrations of Org 42599. Data were fitted by sigmoidal dose-response curves with Hill coefficients of unity and are presented as fold versus basal values for each receptor (mean ± SEM from at least three independent experiments). (**B**) Org41841 rescues Wt hFSHR and mutant A189V (p= 0.002), but is unable to rescue other mutants with mutation at diverse sites in the molecule (SEMs shown). After transfection and incubation in Org41841, cellular response was assessed by a challenge with 100 ng hFSH. As mutants were obtained from different laboratories and were in different expression vectors, controls are shown with the corresponding Wt hFSHR and the empty vector in each case. Figure 3A is reproduced from Newton et al. (2011), with permission of the National Academy of Sciences USA; figure 3B is reproduced from Janovick et al. (2009) with permission of Elsevier Ireland Ltd.

Table 1

Location within the hLHR and clinical phenotype of patients bearing trafficking-defective loss-of-function mutations (in bold letter type) in the *hLHR*. The clinical phenotypes correspond to those described in the original reports.

hLHR mutation	Location	Phenotype	Reference
In-frame 33 bp insertion/ C545Stop (or W491*X*) (compound heterozygous)	Exon 1, signal peptide (33 bp insertion)	Type I LCH: female phenotype and undescended gonads.	(Richter-Unruh et al., 2002; Wu et al., 1998)
V144F	Exon 5, ECD	Type I LCH: female phenotype, slight clitoral enlargement, and inguinal testes.	(Richter-Unruh et al., 2004)
F194V	Exon 7, ECD	Type I LCH: female phenotype, absent virilization, and inguinal testes.	(Gromoll et al., 2002)
C343S/C543R (compound heterozygous)	Exon 11, ECD and TM5	Type I LCH: female phenotype, inguinal testes, abscence of secondary sex characteristics at pubertal age.	(Martens et al., 2002)
A593P	Exon 11, TM6	Type I LCH: female phenotype, absence of pubertal development. In women: primary amenorrhea with pubertal development (full maturation of secondary sexual characteristics).	(Kremer et al., 1995; Martens et al., 1998) (Toledo et al., 1996)
DelL608-V609	Exon 11, TM7	Type I LCH: Female phenotype, pubic hair (Tanner stage 4), testis in labia majora. In women: Normal pubertal development, oligoamenorrhea, infertility, ovarian cysts.	(Latronico et al., 1998)
S616Y	Exon 11, TM7	Type II LCH: male phenotype with micropenis, descended testis, and infertility.	(Latronico et al., 1996)
I625K	Exon 11, TM7	Type II LCH: male phenotype, micropenis and abscence of puberty.	(Martens et al., 1998; Richter-Unruh et al., 2002)

Table 2

Location of trafficking-defective loss-of-function mutations (in bold letter type) within the human FSHR gene and protein, and clinical phenotypes of patients bearing such mutations. The clinical phenotypes correspond to those described in the original reports.

hFSHR mutation	Location	Phenotype	Reference
I160T/R573C (compound heterozygous)	Exon 6, ECD	Partial phenotype of ovarian failure: Normal puberty, secondary amenorrhea, and infertility.	(Beau et al., 1998)
A189V	Exon 7, ECD	Women: Complete phenotype of ovarian failure: Primary amenorrhea, delayed and variable development of secondary sex characteristics, infertility.	(Aittomaki et al., 1996)
		In men: Normal virilization, low-normal to low testicular volume, abnormal semen parameters, fertility.	(Tapanainen et al., 1997)
N191I (simple heterozygous)	Exon 7, ECD	Normal ovarian function and fertility.	(Gromoll et al., 1996)
D224V/L601V (compound heterozygous)	Exon 9, ECD	Partial phenotype of ovarian failure: Normal puberty, primary amenorrhea.	(Touraine et al., 1999)
P519T	Exon 10, eL2	Complete penotype of ovarian failure: Primary amenorrhea, delayed puberty, infertility	(Meduri et al., 2003)