Misrouted cell surface GnRH receptors as a disease aetiology for congenital isolated hypogonadotrophic hypogonadism

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GnRH plays an essential and central role in neuroendocrine control of reproductive function. The GnRH receptor is located on the plasma membrane of gonadotrophs, pituitary cells that synthesize the gonadotrophins LH and FSH. This receptor belongs to the superfamily of G protein-coupled receptors, and is preferentially coupled to the Gq/11 protein; its activation by GnRH analogues stimulates the synthesis and release of LH and FSH. Resistance to GnRH by inactivating (loss-of-function) mutations of the human GnRH receptor leads to distinct forms of sporadic or inherited hypogonadotrophic hypogonadism. Although in vitro expression of a number of these mutated GnRH receptors in heterologous systems has shown that these mutations appear to alter several functions of the molecule, including ligand binding, receptor activation or interaction with coupled effectors, recent observations from our laboratory have challenged this view and indicated that protein misfolding and resultant misrouting is a mechanism that, by itself, may lead to loss of function of the human GnRH receptor. In this review we describe the experimental data that led us to this conclusion and how these studies revealed previously unsuspected features of the mutant human GnRH receptor.

Key words: chaperones/GnRH receptor/hypogonadotrophic hypogonadism/pharmacoperone/receptor mutations

Introduction

GnRH is a decapeptide produced by neurons located in the arcuate nucleus of the mediobasal hypothalamus and in the preoptic area of the anterior hypothalamus. The axons of these neurons project to various regions of the brain, where GnRH acts as neurotransmitter or neuromodulator of reproductive behaviour, or to the median eminence. There the decapeptide enters the portal circulation and reaches its receptor in the plasma membrane of the gonadotroph (Conn and Crowley, 1991; Ulloa-Aguirre and Timossi, 2000). Agonist occupancy of the GnRH receptor (GnRHR) provokes the synthesis and release of the pituitary gonadotrophins LH and FSH, whose coordinated release allows for a highly precise control of gonadal function. Secretion of GnRH to the portal circulation occurs in a pulsatile fashion (Santen and Bardin, 1973; Knobil, 1974). Intermittent exposure of the GnRH receptor to the releasing hormone is important from a functional point of view, since it prevents desensitization (refractoriness) of the gonadotroph to a subsequent stimulus and allows for occurrence of the distinct rates and patterns of synthesis and release of LH and FSH that follow GnRH exposure (Belchetz et al., 1978; Crowley et al., 1985; Conn et al., 1987; Hazum and Conn, 1988).

Decreased synthesis of pituitary gonadotrophins and/or alterations in their episodic release may lead to impaired gonadal function, a condition known in humans as hypogonadotrophic hypogonadism (HH) (Seminara et al., 1998; de Roux and Milgrom, 2001; Ulloa-Aguirre et al., 2004). Some forms of congenital HH result from mutational defects in the synthesis or action of GnRH itself. Although a mutation in the GnRH gene has been reported in the hypogonadotropic hpg/hpg mouse model (Mason et al., 1986), no similar mutation has yet been reported in humans (Weiss et al., 1989, 1991; Bo-Abbas et al., 2003). HH in man is both a clinical and genetic heterogeneous disorder that constitutes one of the most common causes of hereditary hypogonadism (Spitz et al., 1974; Spratt et al., 1987; Chauvain et al., 1988; Dean et al., 1990; Waldstreicher et al., 1996; Georgopoulou et al., 1997; Seminara et al., 1998; de Roux et al., 2001). Sporadic and familial cases of HH with autosomal or X-
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linked modes of inheritance have been described (Santen and Paulsen, 1973; Chaussain et al., 1988; Dean et al., 1990; Seminara et al., 1998; Beranova et al., 2001). When associated with anosmia or hyposmia, the condition is known as Kallman’s syndrome (Kallmann and Schonfeld, 1944; Sparkes et al., 1968; White et al., 1983). The X-linked form of this disease (KAL1) results from mutations in the Kal-1 gene (Meitinger et al., 1983). The X-linked form of this disease, caused by a loss-of-function mutation in the fibroblast growth factor receptor 1 (FGFR1), has been recently described (Dodé et al., 2003). Another X-linked inherited disease that results in congenital adrenal hypoplasia and HH arises from defects in the DAX-1 gene, which encodes a member of the nuclear hormone receptor superfamily (Muscatelli et al., 1994; Habiby et al., 1996; Seminara et al., 1999; Tabarin et al., 2000).

Isolated HH without anosmia or adrenal insufficiency may be truly idiopathic (i.e. due to a still unidentified molecular defect), may arise from mutations in the GPR54 gene (which encodes for a G protein-coupled receptor presumably involved in the secretion of GnRH in mammals) located at chromosome 19p13.3 (Acierno et al., 2003; Bo-Abbas et al., 2003; de Roux et al., 2003; Seminara et al., 2003) or may be provoked by mutations in the human (h) GnRHR gene (de Roux et al., 1997; Layman et al., 1998; reviewed in Kottler et al., 1999; de Roux and Milgrom., 2001; Karges et al., 2003a; Ulloa-Aguirre et al., 2004). To date, 18 inactivating mutations in the hGnRHR gene have been described as causes of HH (de Roux et al., 1997, 1999; Layman et al., 1998; Caron et al., 1999; Pralong et al., 1999; Kottler et al., 2000; Beranova et al., 2001; Costa et al., 2001; Söderlund et al., 2001; Silveira et al., 2002; Karges et al., 2003b; Meyesing et al., 2004). Expression of the majority of these hGnRHR mutants in heterologous systems results in cells that neither bind GnRH agonists nor respond to GnRH stimulation by effector activation. These observations initially suggested that such mutations are associated with alterations in ligand binding, receptor activation or interaction with coupled effectors. Nevertheless, recent observations from our laboratory (Janovick et al., 2002; Leaños-Miranda et al., 2002) have challenged this view, suggesting that protein misfolding and resultant receptor misrouting of otherwise functional receptors is a mechanism that alone may explain the molecular aetiology of this disorder. In this review, we describe the data that led us to this conclusion and the implications of this novel concept for HH and normal hGnRHR function.

The hGnRHR in health and disease

The GnRHR

The GnRHR belongs to the superfamily of G protein-coupled receptors (GPCR), specifically to the family related to the rhodopsin and β-adrenergic receptors (Class A), which is the largest among seven-transmembrane receptors and the best-characterized in terms of its structural and functional characteristics (Kakar et al., 1992; Sealfon et al., 1997; Ulloa-Aguirre and Conn, 1998). Seven transmembrane hydrophobic domains (TMD) oriented roughly perpendicular to the plane of the plasma membrane, with an extracellular amino-terminal, an intracellular carboxyl-terminal and three alternating intra-(I) and extra-(E) cellular hydrophilic loops (L) connecting the TMD, characterize the structure of these receptors (Figure 1a and c) (Ulloa-Aguirre and Conn, 1998; Lu et al., 2002). GPCR can be viewed as ligand-activated switches that activate heterotrimeric guanosine nucleotide-binding proteins (G proteins) (Ulloa-Aguirre and Conn, 1998). Mammalian GnRHR exhibit >85% amino acid identity among the several species that have been cloned (Stojilkovic et al., 1994; Sealfon et al., 1997). Unlike other members of the rhodopsin/β-adrenergic subfamily of GPCR, including the recently described type II GnRHR (Millar et al., 2001, 2003; Neill, 2001, 2002), the mammalian type I GnRHR (hereafter referred to as ‘GnRHR’) exhibits several unique features. These include the reciprocal exchange of the conserved aspartate and asparagine residues in TMD 2 and 7, the replacement of tyrosine with serine in the highly conserved aspartate-arginine-tyrosine (DRY) motif located in the junction of the TMD3 and the IL2 and the lack of the carboxyl-terminal extension into the cytosol (Sealfon et al., 1997) (Figure 1). This latter feature is not exhibited by type II GnRHR from non-mammalian vertebrate species (such as the fish and avian receptors) and non-human primates (Neill, 2002; Millar, 2003) which have a carboxyl-terminal extension; loss of the tail in the GnRHR appears associated with differential physiological regulation including internalization, desensitization and cell surface expression of the receptor in mammals versus pre-mammalian species (Heding et al., 1998; Lin et al., 1998; McArthur et al., 1999). In humans, the GnRHR is located at 4q13.2–3 and consists of three exons and two introns that encode for a 328 amino acid protein (Figure 1b) (Fan et al., 1994; Kottler et al., 1995; Kakar et al., 1997).

The GnRHR receptor is coupled to the trimeric Gq/11 protein, localized in the cytoplasm and associated with the intracellular domains of the receptor (Stojilkovic et al., 1994; Ulloa-Aguirre et al., 1998). Ligand-activation of GnRHR is associated with conformational changes in the TMD of the receptor molecule that extend through the IL, leading to activation of the Gq/11–protein subunit and its dissociation from the Gi/β dimer (Sealfon et al., 1997; Ulloa-Aguirre and Conn, 1998; Ulloa-Aguirre et al., 1998; Lin and Conn, 1999). The activation of the GnRHR–Gq/11 protein complex stimulates the effector enzyme phospholipase Cβ, leading to phosphatidylinositol 4,5-biphosphate hydrolysis and formation of the second messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol. The former messenger diffuses through the cytoplasm, promoting the release of intracellular calcium and the release of gonadotrophins, whereas diacylglycerol activates the enzyme protein kinase C (Conn et al., 1986; Naor, 1990; Stojilkovic et al., 1994; Levi et al., 1998; McArthur et al., 2002). Both IP3-promoted increase in intracellular calcium and activated PKC provoke a series of protein–protein phosphorylation and interactions that include activation of alternate signalling pathways, such as those mediated by mitogen-activated protein kinase cascades (Van Biesen et al., 1996; Ulloa-Aguirre and Conn, 1998; Pierce et al., 2001), which represent an important link for the transmission of signals from the cell surface to the nucleus that eventually lead to regulation of gonadotrophin transcription (Reiss et al., 1997; Naor et al., 2000; Kraus et al., 2001; Harris et al...
Thus, functional integrity of the GnRHR-effector system is required for the expression of a full biological effect in response to GnRH.

**Mutations in the hGnRHR gene**

Structural alterations in key residues of a GPCR molecule or of the G proteins may lead to altered function of the receptor–G protein complex and cause disease. Mutations in sites involved in ligand binding usually result in altered receptors unable to recognize agonists or to become activated (loss-of-function mutations) (Ulloa-Aguirre and Conn, 1998; Ulloa-Aguirre et al., 2003), whereas mutations in sites involved in receptor activation or G protein coupling may lead either to loss-of-function or to constitutive activation (activation in the absence of ligand; gain-of-function mutations) of the receptor molecule (Ulloa-Aguirre et al., 2003). Spontaneous mutations of this latter type have not yet been detected in the GnRHR.

GnRH and its receptor are crucial for pubertal development, sexual maturation and reproductive competence. As with other receptor genes, mutations in the GnRHR gene may lead to abnormal synthesis of the receptor molecule and/or to structural alterations that may potentially alter its functional properties, including ligand binding, coupling to intracellular effectors and intracellular trafficking (Janovick et al., 2002; Maya-Núñez et al., 2002; Bédecarrats et al., 2003a). Resistance to GnRH by loss-of-function mutations in the hGnRHR gene leads to distinct forms of autosomal recessive HH (Figure 1c). Human GnRHR mutations may also occur in individuals with sporadic HH, but with a lower frequency (Bédecarrats et al., 2001). Individuals with HH due to GnRHR mutations exhibit a strikingly wide spectrum of clinical and biochemical phenotypes, including variable alterations in pubertal development, plasma gonadotrophin and sex steroid levels, response to exogenous GnRH administration, and pulsatile pattern of gonadotrophin release (de Roux et al., 1997, 1999;
Caron et al., 1999; Beranova et al., 2001). These alterations usually occur in the absence of anatomical or functional abnormalities of the hypothalamic–gonadotroph axis. Thus, the hypogonadism due to hGnRHR mutations can be complete or partial (reviewed in Kottler et al., 1999; Karges et al., 2003a; Ulloa-Aguirre et al., 2004). The differences between HH phenotypes due to inactivating GnRHR mutations could be related to the particular allelic combination of the co-existing mutations, with the functional activity of a given mutant being more or less severely affected than that exhibited by the other. Nevertheless, different phenotypes may be present within affected kindred bearing the same molecular alteration, thus suggesting that other factors or mechanisms may influence the phenotypic expression of the GnRH-resistant HH (Bédécarrats et al., 2003a,b).

Until recently, 16 mutations in the hGnRHR gene (N10K, T32I, E39K, Q108R, A129D, R139H, S168R, A171T, C200Y, S217R, R262Q, L266R, C279Y, Y284C, L314X, and a splice junction mutation at the intron 1–exon 2 boundary) have been described as causing HH (Table I and Figure 1c) (Ulloa-Aguirre et al., 2004). These mutations are distributed along the entire coding sequence of the receptor, including the NH2-terminus (N10K and T32I), TMD 2 to 7 (E39K, A129D, R139H, S168R, A171T, S217R, C200Y and Y284C), ELs1 (Q108R) and 2 (C279Y) and the IL3 (R262Q and L266R) (Figure 1c), and two (L314X and the intron 1–exon 2 splice site mutation) are truncation mutants. Two hot spots have been identified, the Q108R and the R262Q mutations. More recently, two additional mutations [Q11K (+ N10K) in the NH2-terminus, and P320L in the TMD7] in the hGnHR gene causing HH have been described (Meysing et al., 2004). Expression of original 16 mutated hGnRH receptors in heterologous cell systems has shown that these mutations may alter several functions of the molecule including ligand binding, receptor expression at the cell surface and/or signal transduction, consistent with the view that the mutation interferes with the functional ability of the receptor to bind ligand or couple effector

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genotype</th>
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<tr>
<td>Q108R</td>
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<td>Decreased ligand binding; NME*89</td>
<td>de Roux et al., 1997</td>
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<td>Decreased IP3 production</td>
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<td>Caron et al., 1999</td>
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<td>de Roux et al., 1999</td>
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<tr>
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<td>Pralong et al., 1999</td>
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<td>Homozygous</td>
<td>Reduced ligand binding; Absent IP3 production NME*89</td>
<td>Meysing et al., 2004</td>
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* NME, normal membrane expression  † Mutations in the same allele.
(Ulloa-Aguirre et al., 2004). Nevertheless, our recent studies indicate that many loss-of-function mutations of the hGnRHR, initially thought to impair ligand binding or interaction with coupled effectors as the primary defect, actually result from protein misrouting (Janovick et al., 2002; Leãños-Miranda et al., 2002; Maya-Nuñez et al., 2002). Before describing the particular genetic and pharmacological approaches that led us to consider membrane receptor misrouting as the specific disease aetiology for a number of the naturally occurring human GnRHR reported to date, let us briefly review how defectively nascent proteins may lead to disease.

### Protein folding and disease

Synthesis and processing of secretory and membrane proteins occur in the endoplasmic reticulum (ER). This cellular organelle synthesizes and assembles nearly 100 000 proteins, including heteromeric molecules; in the lumen of the ER, protein molecules fold and oligomerize, disulphide bonds are formed, and N-linked oligosaccharides are added. These processes are regulated at the transcriptional and translational levels by multiple factors including hormones and growth factors. According to current models of protein folding, as proteins are synthesized in the ER, they fold and adopt a distinct conformation that allows the molecule to acquire a stable, active structure potentially compatible with ER export (Belloti et al., 1999; Brooks, 1999; Radford and Dobson, 1999; Sanders and Nagy, 2000). Quality control mechanisms then recognize specific shapes resulting from protein wriggling, hence determining the routing, intracellular trafficking and metabolic fate of the nascent protein within the cell (Helenius, 2001; Ellgaard and Helenius, 2001; Cahill and Cahill, 2002). The quality control system includes a family of proteins called ‘chaperones’ which help to fold nascent proteins in the ER and prevent aggregation in a crowded cell environment or prevent misfolded protein molecules from making incorrect interactions with other molecules (Ellgaard, 1999; Morello et al., 2000a; Hartl and Hayer-Hartl, 2002; Luque et al., 2002). If correction of misfolding fails, then the incorrectly manufactured protein is targeted for destruction in proteosomes. In some GPCR, molecular chaperones are also involved in surface expression of the newly synthesized or recycled receptor (Brady and Limbird, 2002). For example, in the nematode Caenorhabditis elegans, the odr-4 gene encodes for a regulatory molecule specifically expressed in chemosensory neurons where the chaperone assists in folding and/or targeting of the ODR 10 receptor to olfactory cilia (Dwyer et al., 1998), whereas in Drosoˆphila melanogaster, absence of the chaperone Nina A (neither inactivation nor afterpotential A) leads to rhodopsin 1 ER accumulation and eventually to its degradation (Schneuwly et al., 1989; Shieh et al., 1989; Colley et al., 1991; Liu et al., 1995). Nina A and rhodopsin complex also aid in proper folding and transport of mature receptor to the cell membrane (Baker et al., 1994). Several GPCR, including the LH/choriogonadotrophin receptor, the FSHR (Rozell et al., 1998) and the V2-vasopressin receptor (Morello et al., 2001), interact with calnexin, a molecular chaperone (Helenius et al., 1997); an abnormal interaction between calnexin and the R337X mutant of the V2 receptor is apparently responsible for the absence of cell surface expression of the mutant molecule, a condition that leads to X-linked nephrogenic diabetes insipidus (Morello et al., 2001).

Protein mutations may result in misrouting of newly synthesized proteins and consequently to their premature or inappropriate processing and rapid degradation. In principle, this could also result in abnormal accumulation in the ER. Accumulation and aggregation of misfolded proteins are presumably responsible for some neurodegenerative diseases such as early onset familial Alzheimer disease and Parkinson disease (Forloni et al., 2002; Forman et al., 2003), as well as for early onset catacaRas (Sandilans et al., 2002; Kosinski-Collins and King, 2003). Diseases caused by cell surface protein mislocalization of otherwise functionally competent molecules include some forms of familial hypercholesterolaemia (Hobbs et al., 1990), retinitis pigmentosa (Sung et al., 1994; Colley et al., 1995; Dryja and Li, 1995; Deretic et al., 1996; Li et al., 1996), cystic fibrosis (Deming et al., 1992; Welsh and Smith, 1993) and diabetes insipidus (Birnbaumer, 1999; Knoers and Deen, 2000; Bichet et al., 2001). Several mutations in the low density lipoprotein (LDL) receptor involve defects in trafficking and/or processing of the receptor (Hobbs et al., 1990; Patel et al., 1998). In cystic fibrosis, the ∆F508 mutation (which is found in ~70% of patients with this condition), leads to retention and rapid degradation of the incompletely processed cystic fibrosis transmembrane conductance regulator (CFTR) in a compartment proximal to the Golgi apparatus; this prevents cell surface expression of the channel protein and consequently provokes loss of cyclic AMP-regulated chloride transmembrane conductance (Welsh and Smith, 1993). In nephrogenic diabetes insipidus, urine is not concentrated due to resistance of the kidney to arginine-vasopressin or to defects involving the arginine-vasopressin-responsive aquaporin-2 water channel (Knoers and Deen, 2001). When expressed in vitro in heterologous systems, most V2-vasopressin receptor mutations exhibit intracellular trapping of the receptor molecules that are then unable to reach the cell membrane (Morello and Bichet, 2001). Similarly, mutations of the aquaporin-2 water channel can provoke misrouting of the protein, preventing its cell membrane expression (Tamarappoo and Verkman, 1999; Knoers and Deen, 2001). In the case of retinitis pigmentosa (a disease characterized by retinal degeneration, subsequent night blindness and eventual total blindness), mutations in the gene encoding rhodopsin may result in defective molecules that misfold and accumulate in the ER (Sung et al., 1994); in particular, mutations in the carboxyl-terminus of rhodopsin may cause defects in receptor trafficking to the outer segment of the rod cell (Deretic et al., 1996). A final example of membrane receptor misrouting as specific disease aetiology is the E50K mutation in the hGnRHR gene. Patients bearing this particular genetic defect express a severe phenotype of HH (Söderlund et al., 2002). When the mutant E50K-defective receptor was genetically modified by deleting the amino acid K316 (a deletion that enhances membrane expression of the wild type (WT) human receptor (Arora et al., 1999; Maya-Nuñez et al., 2000), both membrane targeting and function of the E50K mutant were rescued as disclosed by immunolocalization of the receptor, ligand binding, and measurement of coupling to an effector system (Figure 2) (Maya-Nuñez et al., 2002). This finding led to the consideration that misrouting or mis-positioning of an otherwise competent receptor was a more satisfactory and alternative explanation for the inactivity showed by this particular mutant GnRH receptor, and led us to consider the possibility that some other naturally occurring mutant hGnRHR molecules may
actually bear conformational defects sensitive to functional rescue by exogenous means.

Several approaches have been applied to salvage defective proteins. Among these are the use of physical methods (Denning et al., 1992; Brown et al., 1997; Matsuda et al., 1999; Zhou et al., 1999), non-specific protein stabilizing agents (such as polyols and sugars) (Sato et al., 1996), genetic modification of mutant proteins (‘genetic rescue’) (Cheng et al., 1995; Schülein et al., 2001; Mayana-Núñez et al., 2002) and use of template molecules or pharmacological chaperones (‘pharmacoperones’) that correct errors in folding and restore activity by correct routing (‘pharmacological rescue’) (Zhou et al., 1999; Morello et al., 2000a; Petäjä-Repo et al., 2002).

Studies on the biosynthesis and localization of the CFTR ΔF508 mutant in cystic fibrosis have shown that the mutant protein is not processed correctly and, accordingly, is not delivered to the cell surface plasma membrane (Welsh and Smith, 1993; Denning et al., 1992). Nevertheless, expression of this mutant in Xenopus oocytes and in Sf9 insect cells (which are usually maintained at lower temperatures than mammalian cells) led to detection of chloride channel activity owing to the processing sensitivity of nascent proteins to temperature (Denning et al., 1992). Moreover, incubation at reduced temperatures (20–30°C) reverted processing of the CFTR mutant towards the wild type receptor, allowing the cAMP-regulated chloride channel to be expressed at the cell surface membrane (Denning et al., 1992). Similarly, incubation of stable CFTR ΔF508 transfectants with glycerol (a polyhydric alcohol that stabilizes protein conformation) provoked the accumulation of functional ΔF508 protein and an increase in whole cell Cl⁻ conductance (Sato et al., 1996). Nevertheless, while such approaches can rescue incompletely processed mutants, these are predictably non-specific, and therefore of limited therapeutic value. Genetic approaches in which further modifications are introduced to an already defective protein have been used to rescue function of conformationally abnormal molecules. These approaches either overexpress or stabilize extant molecules rendered unstable by genetic defects or effect corrected trafficking by adding targeting sequences. Rescue of the mislocalized CFTR ΔF508 mutant, which otherwise retains significant phosphorylation-regulated Cl⁻ channel activity, can be achieved by overexpression of the ΔF508 mutant regulator, an effect that results in the escape of limited amounts of the mutant protein to the plasma membrane (Cheng et al., 1995). Introduction of additional cysteine residues in the extracellular domains of GPCR may potentially provoke formation of additional disulphide bonds or impair formation of a conserved bond, leading to binding- or trafficking-defective receptors (Sung et al., 1991; Schülein et al., 2001; Janovick et al., 2002). In the former situation (binding-defective) it is possible to rescue receptor function by introducing a second site suppressor mutation that abrogates formation of additional disulphide bonds (Schülein et al., 2001). A third example of genetic rescue is the GnRHR E90K mutant described above whose...
plasma membrane localization and function may be rescued by deleting the amino acid K191 (Figure 2) (Maya-Núñez et al., 2002). Genetic techniques are limited as therapeutic approaches since, were it possible to correct the gene sequence, the primary error could be directly addressed by this means. Nevertheless, these findings are of considerable interest since they lead to the possibility that the mutation itself does not significantly alter the ligand binding or effector coupling capability of the defective molecule, particularly in the case of trafficking-defective proteins.

The observations that binding- or trafficking-defective proteins that are normally retained by the ER quality control system and eventually degraded may be functionally rescued by physical, chemical and genetic interventions led to the design and development of pharmacological agents that may rescue abnormally folded proteins or receptor molecules having intrinsically low maturation efficiencies. Given that small ligand molecules may influence folding and organelle targeting of some proteins and that antagonists may prevent down-regulation of GPCR, it was thought that small, cell-permeable antagonists could stabilize specific conformation of mutant AVP V2R and restore cell surface membrane expression (Morello et al., 2000b). Treatment of cells expressing mutant AVP V2R with two non-peptidic V2R antagonists (SR121463A and VPA-985) promoted proper folding, maturation and targeting of mutant receptors to the cell surface (Morello et al., 2000b).

For the human δ opioid receptor, for which only a fraction (~40%) is normally transferred to the cell membrane (Petaja-Repo et al., 2000, 2001), both membrane-permeable agonists and antagonists facilitated post-translational processing and increased export of the ligand-stabilized receptor from the ER to the cell surface (Petaja-Repo et al., 2002).

Desirable characteristics of molecules that may potentially serve as pharmacoperones and effect pharmacological rescue of mutant proteins include: (i) specificity to the molecule being rescued, (ii) ability to enter the target cell, reach the ER, and remain stable long enough to bind and stabilize the nascent protein and (iii) ability to dissociate from the molecule rescued (or, at least, not compete with the natural ligand binding site) after the arrival to the appropriate target locus. In this setting, the cell-permanent GnRH antagonist IN3 was chosen as a potentially useful pharmacological chaperone to rescue function of conformationally abnormal hGnRH receptors.

### Misrouted hGnRHR as disease aetiology for isolated HH

Figure 1c shows the distribution of the 17 loss-of-function hGnRHR mutations detected in patients with isolated HH. When expressed in heterologous cell systems, some mutants are completely non-functional (E90K, A129D, R139H, S168R, A171T, C200Y, S211R, L266R, C279Y, L314X, and P320L) while others retain some degree of function (N10K, Q11K (+N10K), T32I, Q106R, R262Q, and Y284C) (Table I) (Kottler et al., 1999; de Roux et al., 2001; Karges et al., 2003a; Meysing et al., 2004; Ulloa-Aguirre et al., 2004). The commonly held belief that these mutations interfere with ligand binding or receptor interaction with G proteins has been recently questioned by studies showing that both membrane expression and function of a number of these hGnRHR mutants are sensitive to rescue by pharmacological chaperones (Janovick et al., 2002, 2003a; Leaños-Miranda et al., 2002). Thus, the underlying defect of such defective receptors resides on misfolding, which impedes the receptor protein (which is otherwise functionally competent in terms of its ligand-binding and effector-coupling abilities) to reach its proper locus at the cell surface.

As described above, when the hGnRHR E90K mutant was expressed in COS-7 cells, it did not exhibit even a modest level of binding (Maya-Núñez et al., 2002; Janovick et al., 2002, 2003a) and, in sharp contrast to the wild-type GnRHR, did not show GnRH agonist-stimulated inositol phosphate (IP) turnover. Likewise, seven other hGnRHR mutants and the W205X mutant, a laboratory manufactured hGnRHR truncated at position 205 used as a negative control, showed no stimulation of IP turnover in response to the GnRH agonist Buserelin, whereas five other mutants showed greatly reduced stimulation of IP production (Figure 3) (Leaños-Miranda et al., 2002). The potential benefits of a peptidomimetic, cell membrane-permeant antagonist of GnRH, (2S)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]-2-(3,5-dimethylphenyl)-1H-indol-3-yl]-N-(2-pyridin-4-yl ethyl)-propan-1-amine (or ‘IN3’) [synthesized by Drs Wallace T.Ashton and Mark Goulet, Merck and Company, Rahway, NJ, USA] (Ashton et al., 2001a–c), as a pharmacological agent that could serve as a template for misfolded GnRHR and thereby effect rescue on naturally occurring hGnRHR mutants, was then experimentally evaluated. For 11 of the 13 HH mutants tested, exposure to 1.75–4.50 μmol/l concentrations of IN3 at the time of transfection resulted in both Gq/11 coupling and highly specific ligand binding (Figure 4a and b) (Janovick et al., 2002; Leaños-Miranda et al., 2002), demonstrating that the effect of the mutational error leading to intracellular misrouting was either completely or partially corrected, except in the case of S168R, S211R and the truncation mutant W205X (which was predictably unrescuable by IN3 as it is a substantially incomplete piece of the
Ligand specificity of the rescued E90K receptor (Janovick et al., 2002) was indistinguishable from published values for the WT hGnRHR (Vrecl et al., 1998; Ashton et al., 2001b). GnRH agonists (Buserelin and Leuprolide) and antagonists (Antide, Nal-Glu, Nal-Arg and Cetrorelix) tested were recognized with high fidelity and the varying potencies characteristic of the WT hGnRHR (Myburgh et al., 1998; Vrecl et al., 1998). Further, selected irrelevant compounds (which normally do not bind the WT receptor) evoked no IP response (Janovick et al., 2002). Peptide antagonists, which cannot permeate cells and that were designed by chemical analogy to agonists (and are therefore expected to bind to the same site), were unable to rescue HH deletion mutants or rat GnRHR cysteine mutants (see below) (Janovick et al., 2002). The antagonists selected were weak (DPhe2, DPhe6-GnRH), intermediate (‘Nal-Glu’) and high (Cetrorelix) affinity binders. The inability of such specific non-permeant GnRH antagonists to rescue HH deletion mutants or cysteine mutants ruled out the possibility that IN3 had stabilized the structure of a scarce receptor population that had reached the plasma membrane, thereby promoting their accumulation over time.

The A171T hGnRHR mutant, which presumably leads to receptor stabilization in an inactive conformation (Karges et al., 2003a), has more recently been tested for pharmacological rescue; exposure of COS-7 cells transiently expressing the A171T mutant to IN3 increased Buserelin-stimulated inositol phosphates production to approximately one-fifth of the maximal response observed for the WT receptor (Figure 5).

A number of laboratory-manufactured rat GnRHR with terminal truncations, internal deletions or mutations at sites in which a cysteine residue normally appears (this amino acid is associated with maintenance of the tertiary structure of the receptor) have been additionally examined (Janovick et al., 2002). When varied substitutions were made at the same locus (i.e. C278A, C278V, C278T, C278M), there were differences in the ability to effect rescue. The mutants resulting from the more bulky substituents (C278V and C278T) were apparently unable to reach a conformation necessary for rescue with a low dose of IN3. Therefore, the expression of such variants on the plasma membrane also appeared variable in the absence of IN3. Substitution of the bulky W279 (adjacent to C278) with alanine (W279A) also produced an inactive mutant that was rescued by IN3 (Janovick et al., 2002). Of particular interest are the double mutants, C278W/W279C (exchange

![Figure 4](https://academic.oup.com/humupd/article-abstract/10/2/177/617171/1 by guest on 15 March 2019)

**Figure 4.** Stimulation of inositol phosphate (IP) production by Buserelin (10⁻⁷ mol/l) (a) and binding of [¹²⁵I]-labelled Buserelin (b) in COS-7 cells transiently transfected with the hGnRHR wild-type (WT), naturally occurring hGnRHR mutants or the pcDNA3.1 vector. Cells were cultured in the absence (−) or presence (+) of 1.75 μmol/l concentration of IN3 for 27 h.

![Figure 5](https://academic.oup.com/humupd/article-abstract/10/2/177/617171/1 by guest on 15 March 2019)

**Figure 5.** Stimulation of inositol phosphate (IP) production by 10⁻⁷ mol/l Buserelin in COS-7 cells transiently transfected with the hGnRHR A171T mutant and incubated in the absence (−) or presence (+) of IN3. The response of cells expressing the wild-type (WT) hGnRHR or the hGnRHR E90K mutant are also shown for comparisons.
of WT sequence), C278V/W279V and C278A/W279A. Although the first two were only modestly rescuable by high dose IN3, the latter could be restored to full function. The difference, again, appeared to reflect the significant steric constraints associated with the larger amino acid residues, valine, tryptophan and cysteine. Alanine, in contrast, allows for more steric freedom owing to its smaller size; it better accepted the folding template’s adjustment of configuration, resulting in the highest level of IP production (Janovick et al., 2002). Mutation at C229 produced a defect from which rescue could not be effected, suggesting that this residue may have other roles in receptor interaction with other molecules. Alternatively, this mutation may result in an unrescueable configuration, with the possibility that other pharmacoperones may effect rescue.

Finally, the ability of IN3 to rescue a shortened carboxyl-terminal truncation rat GnRHR mutant [des(325–327)], which showed virtually no response to GnRH when expressed in culture cells, was examined (Janovick et al., 2002). Deletion of these terminal residues resulted in loss of GnRH agonist-evoked IP production; nevertheless, much of this activity could be recovered by IN3. Removing a larger (12 amino acid) sequence from the carboxyl-terminus yielded a mutant ([des316–327]) that could not be rescued, although as many as four amino acids could be removed from the third intracellular loop ([des237–241] GnRHR) and rescue, albeit partially, still effected (Janovick et al., 2002). Although the ability of IN3 to rescue the human GnRHR L314X mutant has not been tested yet, the results obtained with the rat GnRHR [des(316–327)] receptor predict that the naturally occurring L314X truncated receptor will not be rescued by pharmacological means.

The ability of different GnRH peptidomimetics to rescue defective GnRHR mutants has been more recently assessed (Janovick et al., 2003a). This study included five laboratory manufactured ‘loss-of-function’ rat GnRHR [des (325–327) GnRHR (the shortest rat GnRHR carboxyl-terminal truncation mutant that resulted in receptor loss-of-function), des(237–241) and des(260–265) rat GnRHR (two non-functional deletion mutants), C229A and C278A rat GnRHR (two non-functional Cys mutants)] and 13 naturally occurring full-length hGnRHR point mutants (N94K, T31I, E80K, Q108R, A129D, R139H, S168R, C200Y, S217R, R262Q, L266R, C278V/W279V, and C278A/W279A). The 10 peptidomimetics assessed as potential rescuers were from three different chemical classes [indoles, quinolones (Merck and Company), and erythromycin-derived macrolides (Abbott Laboratories, USA)], and were originally developed as GnRH peptidomimetic antagonists. These particular structures were selected considering their predicted ability to permeate the cell membrane and interact with a defined affinity with the GnRHR. All peptidomimetics studied with an IC50 value (for the human GnRHR) <2.3 nmol/l displayed a measurable efficacy in rescuing GnRHR mutants, and within a single chemical class, this ability correlated to these IC50 values (Janovick et al., 2003a). Erythromycin-derived macrolides with IC50 values as high as 669.5 nmol/l showed efficacy as pharmacoperones. Further, the ability of a given compound to rescue a defective receptor was a reasonable predictor of its ability to rescue others, even across species lines, although particular mutants (human S168R and S217R GnRHR, and rat des(260–265) and C229A GnRHR) could not be rescued by any of the drugs tested.

The locus to which mutant GnRHR might be misrouted is unknown; nevertheless, it would not be surprising to find that different mutants (or laboratory-manufactured defective receptors) might be routed to entirely different compartments. In fact, we specifically noted that misrouting may even include the positioning of a mutant receptor in the plasma membrane in such a position that makes the receptor unreachable for the ligand or unavailable to the G proteins or the effector enzymes. This view, however, does not exclude the possibility that some mutants, exhibiting either low responsiveness or refactoriness to pharmacoperone treatment, may in fact bear structural defects involving particular microdomains critical for ligand binding, receptor activation and/or effector coupling (Arora et al., 1997; Sealfon et al., 1997) but not those essential for proper protein folding and intracellular routing. For example, in the case of the R139H mutation, which affects the conserved DRS motif located in the junction of the TMD3 and the IL2, in vitro pharmacological treatment corrected expression and coupling efficiency of the mutant hGnRHR to a modest extent; it is well established, however, that structural integrity of this particular motif is essential for proper receptor activation and effector coupling in a number of GPCR (Ulloa-Aguirre and Conn, 1998), including the hGnRHR (Arora et al., 1997). In the case of the S168R mutation (in TMD4) as well as the A129D and S217R mutations (in TMD3 and 5 respectively), which are refractory to pharmacoperone treatment, the abnormal substitutions may disrupt potential interactions between these or adjacent helices and severely impair agonist binding (Javitch et al., 1995; Unget et al., 1997; Caron et al., 1999; de Roux et al., 1999). An alternative possibility is that these unresponsive mutants cannot be refolded and exported from the ER to the cell surface, even in the presence of pharmacoperones.

The A171T mutant, which has been reported to exist in a stable, but inactive, conformation (Karges et al., 2003b), is only partially rescued by IN3. This latter observation suggests that incubation with the pharmacoperone may impede the additional hydrogen bond formation between TMD3 and TMD4 proposed by Karges et al. (2003b). Nevertheless, additional studies are needed before claiming that the A171T substitution modifies the intracellular trafficking of the hGnRHR receptor.

One interesting observation is that treatment with the pharmacoperone IN3 also increased the expression level of WT hGnRHR (Janovick et al., 2002, 2003b; Lealos-Miranda et al., 2002, 2003). This observation suggests that, as with other membrane receptors (e.g. the human δ opioid receptor; Petjä-Jepo et al., 2001), a large portion (nearly 50%) of the WT hGnRHR is intentionally inefficiently processed by the cell, retained in the ER and eventually degraded. In this setting, incompletely processed receptors may function as a reserve pool of molecules that can be called upon when needed quickly (e.g. under IN3 exposure). This view is supported by the observation that the pharmacoperone IN3 does not increase the functional expression of hGnRHR ΔK191, catfish GnRHR carboxyl-terminal tail/hGnRHR chimera (Figure 2), rat GnRHR (in which K191 is absent), and rat GnRHR chimeras bearing the catfish GnRHR carboxyl-terminal tail or several carboxyl-terminal tail-truncated fragments (Janovick et al., 2003b), suggesting that these receptors exhibit high intrinsic maturation efficiency and membrane expression levels while the WT hGnRHR is routed to the functionally active site on the plasma membrane at a lower percentage. The intrinsically lower expression of the hGnRHR may additionally reflect a relatively new evolutionary development for this receptor, as deletion of the
primate-specific K191 or addition of the piscine carboxyl-terminal tail increases plasma membrane expression yet produces a modified receptor that does not increase plasma membrane expression by pharmacological means (Janovick et al., 2003b).

The effects of human GnRHR mutants on wild-type GnRHR function

The GnRHR was the first GPCR shown to activate upon dimerization (Conn et al., 1982a,b; Cornea et al., 2001), an event that currently seems to be a general feature of this superfamily of membrane receptors (Rios et al., 2001; Angers et al., 2002). Some GPCR are monomeric in the membrane and dimerize upon ligand binding, whereas others dimerize as they are synthesized in the ER, an apparent requisite for correct targeting to the cell surface (Rios et al., 2001; Angers et al., 2002; Cornea and Conn, 2002; Patel et al., 2002). Intracellular association of GPCR as homo- or heterodimers could lead, in principle, to either cell surface targeting (a dominant-positive effect, as is the case for metabotropic γ-amino-butyric acid B1 and B2 receptors) (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) or to intracellular retention of the complex (dominant-negative effect, as appears to be the case for the V2-vasopressin, platelet-activating factor, and CCR5 chemokine receptors) (Benkirane et al., 1997; Zhu and Wess, 1998; Le Gouill et al., 1999). Given that truncated variants of the V2-vasopressin receptor (Zhu and Wess, 1998), the hGnRHR (Grosse et al., 1997) and the D2 and D3-dopamine receptors (Karpa et al., 2000; Lee et al., 2000) may interfere with the cell surface expression of their corresponding WT receptors (presumably due to association in the ER and misrouting of the resulting complex), we tested the hypothesis that some of the human GnRHR mutants, presumptively bearing folding defects as disclosed by their sensitivity to pharmacoperones, may affect WT GnRHR function (Lean-Años-Miranda et al., 2003). To this end, we analysed the effects of eight naturally occurring hGnRHR mutants [E90K, A126D, R139H, S168R, C200Y, S217R, L266R, and C279Y, which when expressed individually showed a complete inability to stimulate inositol phosphate production in response to GnRH agonist (Table I)] on plasma membrane expression and effector coupling of the WT hGnRHR. Co-expression of these receptor mutants with their WT counterpart inhibited both WT GnRHR-mediated agonist binding and intracellular signalling in a dose-dependent manner and with specificity for individual mutant cDNA (Figure 6). Maximal responses to GnRH agonist declined by 16–17% in cells expressing a relative excess (3–8-fold) of GnRHR mutant cDNA and the inhibition was more pronounced when provoked by mutants bearing substitutions located across the TMD 3 to 6 of the receptor (S168R, S217R, and L266R). Further, in cells co-expressing the hGnRHR mutants and the WT receptor, exposure to IN3 partially or completely ablated the negative effect

Figure 6. Dominant negative action of disease-causing hGnRHR mutants. (a) Inhibition of GnRH agonist-stimulated inositol phosphate (IP) production by co-expression of the different hGnRHR mutants and the wild-type (WT) receptor; COS-7 cells were transiently co-transfected at a 8:1 hGnRHR mutant to WT hGnRHR ratio. (a) Maximal IP production in response to 10−7 mol/l Buserelin (mean ± SEM of at least three independent experiments in triplicate incubations). (b) Concentration–response curves at the indicated Buserelin doses (results are representative of three or four independent experiments). Similar trends were found when cells were co-transfected at a 1:1 or 3:1 ratio (Lean-Años-Miranda et al., 2003). Inset: Inhibition of specific [125I]-labelled Buserelin binding (mean ± SEM of quadruplicate incubations) by co-expression of hGnRHR mutants and the WT hGnRHR; COS-7 cells were transiently co-transfected with each hGnRHR mutant and the WT receptor at a 4:1 ratio. *P < 0.05 versus WT and pcDNA3.1 vector; **P < 0.01 versus WT and pcDNA3.1; ***P < 0.001 versus WT and pcDNA3.1.
of the mutants on WT receptor function; the function of the WT receptor complexed with IN3-sensitive mutants (E69K, C200Y, or L268R) recovered to levels above those observed for the WT receptor alone, suggesting that the pharmacoperone interacted with and successfully rescued both the mutant and the WT receptor species (Leanos-Miranda et al., 2003).

Overall, these findings suggest that the impaired WT GnRHR function may be due to intermolecular interactions between the WT receptor and the naturally occurring mutants. In fact, it has been shown that a number of mutant GPCR or splice variants may potentially act as negative (Zhu and Wess, 1998; Benkirane et al., 1997; Grosse et al., 1997; Karpa et al., 2000) or positive (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Ji et al., 2002; Lee et al., 2002) regulators of membrane receptor expression and function via formation of oligomers that hamper or facilitate proper delivery and/or insertion of the native receptor to the cell surface. The observation that the hGnRHR mutants displayed dominant-negative effects when coexpressed with the WT receptor, but not with genetically modified hGnRHR intrinsically exhibiting high maturation efficiencies [such as the hGnRHR-ΔK191 and the hGnRHR chimera bearing the catfish GnRHR carboxyl-terminal tail] or the WT rat GnRHR (Janovic et al., 2003b), suggests that as with other GPCR (Benkirane et al., 1997; Zhu and Wess, 1998; Lee et al., 2000; Sullivan et al., 2000) conformational variants of GnRHR are prone to associate and form complexes whose fate will depend on the particular conformation adopted by the complexed proteins. The data additionally suggest that the dominant-negative effect of the mutants on WT GnRHR function requires intrinsic low plasma membrane expression of the WT GnRHR species that co-evolved with the dominant-negative effect. Although desirable to do so, we have been unable to use microscopic techniques to monitor the intracellular routing and membrane targeting of the GnRH receptor because the use of green fluorescent protein derivatives of this particular receptor requires the presence of a catfish tail spacer (Lin et al., 1998; Brothers et al., 2003), which, itself, significantly influences receptor expression (Maya-Núñez et al., 2000). Further, recent observations in our laboratory (Brothers et al., 2003) indicate that early missense sequences required for epitope-tagging [e.g. haemagglutinin (HA) influenza virus epitope tag] can rescue particularly conformationally defective hGnRHR mutants. In fact, the previous use of the HA tag to identify plasma membrane expression of some mutant hGnRHR [e.g. T12I, Q106R, C200Y and C278Y (Beranova et al., 2001), and N10K (Costa et al., 2001)] obscured recognition that such hGnRHR mutants bear conformational defects that may potentially hamper cell-surface transport.

As previously mentioned, individuals with HH due to loss-of-function mutations in the GnRHR are either compound heterozygous or homozygous for the mutation (Table I). Carriers of a mutant allele usually exhibit normal gonadotrophin levels as well as normal responsiveness to exogenous GnRH stimulation and reproductive competence (Caron et al., 1999; Kottler et al., 1999). It is possible that these carriers express both WT and mutant receptors at levels compatible with the expression of a normal phenotype or, alternatively, that the expression levels of the WT receptor, albeit reduced by the negative effect of the mutant receptor, may be otherwise sufficient to mediate physiological effects.

Conclusions

The observation that naturally occurring mutants of the GnRH receptor may be rescued by pharmacoperones suggests that these disease-related genetic defects are associated with structural changes that result in misrouting, rather than loss of the ability to bind ligand or activate effectors, normally coupled to the WT counterpart. For this reason, it is interesting to consider both the biochemical and clinical classes into which such mutations fall. At the time of writing, there are 18 mutants known to us to cause isolated HH. Of these, two are missing large sequences: one being a truncation of all amino acids between 314 and the carboxyl terminal amino acid 328 (Kottler et al., 2000) and the other, a deletion mutant missing exon 2 (Silveira et al., 2002). It is reasonable to assume that such a large sequence omissions would have a dramatic effect on the receptor structure. The remaining mutations are subtler, involving only a single amino acid. Of these, three involve loss (two occurrences) or gain (one occurrence) of a cysteine residue, an amino acid known to form bridges associated with the formation of third order structure of proteins. Disruption of required bridges or formation of inappropriate bridges would also be significantly disruptive to the structure of the protein. One of the recently reported mutations (Meyting et al., 2004) is associated with the loss of a proline at amino acid 320, which is replaced by leucine. 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. Although this is a hydrophobic-for-hydrophobic substitution, the abrupt turn is likely requisite for routing or activity and cannot be corrected by the pharmacoperones. The remainder, 12 mutants, are surprisingly modest changes in a single charge. Ten of these 11 mutations involve lysine (three occurrences, positively charged), arginine (six occurrences, positively charged) or aspartate (one occurrence, negatively charged). Introduction of (even minor) charge modifications appears sufficient to create altered structure. Of interest, none of the reported mutations is of a conservative nature, in which alanine replaces a glycine or threonine replaces a serine; in each case adding a single carbon without modifying the net charge. Curiously, there are no examples of simple hydrophobic-for-hydrophobic exchanges (valine-for-alanine, for example), positive-for-positive (lysine for arginine) exchanges or negative-for-negative (aspartate-for-glutamate) exchanges. Such exchanges may occur, of course, but be clinically silent or, in the alternative, the phenotype may not survive.

Based on the functional characteristics of the hGnRHR mutants described above and their particular response to genetic and pharmacological approaches intended to correct errors in folding and restore intracellular routing, we propose the following classification of the naturally occurring hGnRHR mutants reported to date:

Type I. Defective binding, processing and/or function: S168R and S217R.

Type II. Defective intracellular transport by misfolding:

IIa. Misfolding and abnormal trafficking: E69K.

IIb. Misfolding with potentially increased degradation: N10K, T32I, Q106R, R262Q and Y284C.

Type III. Defective binding, processing and/or function with defective intracellular transport by misfolding: A125D, R139H, C200Y, L268R, C278Y and A171T.
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In this scenario, the L314X truncated receptor and the deletion mutant missing exon 2 would fall into the Type I category. In those mutants belonging to group type Ib, increased degradation as a result of misfolding might explain their limited function in the in vitro untreated state.

Given that rescue molecules need not be agonists or antagonists, it is likely that extant chemical archives may already keep valuable compounds potentially useful as pharmacoperones for misfolded hGnRHRs (or for other diseases). Such compounds might have been overlooked in high-throughput screens for agonism or antagonism; their use might actually be advantageous therapeutically since they would not interfere with the active site and consequently might not need to be removed from the receptor to allow for agonistic activation and normal physiological function. In this setting, GnRH agonists, albeit able to bind the defective receptor tightly and long enough to correct the misfolding, would be poor rescuers since they may potentially lead to receptor desensitization once receptor rescue is effected. The finding that some degree of misfolding, which makes the defective receptor a potential target for novel, pharmacoperone-based therapeutic strategies, is of paramount importance, particularly considering that the frequency of hGnRHR mutations in familial HH may be actually more common than previously recognized (Benerova et al., 2001).

Finally, the observation that a significant fraction of the WT hGnRHR is incompletely processed to the cell surface membrane is conceptually important. Apparently, this particular feature occurred recently in evolution (Janovick et al., 2003b), perhaps to accompany the more complicated requirements for primate cyclicity and reproduction.

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