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INVITED REVIEW

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Restoring function to inactivating G protein-coupled receptor variants in the hypothalamic–pituitary–gonadal axis[1]

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Abstract

G protein-coupled receptors (GPCRs) are central to the functioning of the hypothalamic–pituitary–gonadal axis (HPG axis) and include the rhodopsin-like GPCR family members, neurokinin 3 receptor, kappa-opioid receptor, kisspeptin 1 receptor, gonadotropin-releasing hormone receptor, and the gonadotropin receptors, luteinizing hormone/choriogonadotropin receptor and follicle-stimulating hormone receptor. Unsurprisingly, inactivating variants of these receptors have been implicated in a spectrum of reproductive phenotypes, including failure to undergo puberty, and infertility. Clinical induction of puberty in patients harbouring such variants is possible, but restoration of fertility is not always a realisable outcome, particularly for those patients suffering from primary hypogonadism. Thus, novel pharmaceuticals and/or a fundamental change in approach to treating these patients are required. The increasing wealth of data describing the effects of coding-region genetic variants on GPCR function has highlighted that the majority appear to be dysfunctional as a result of misfolding of the encoded receptor protein, which, in turn, results in impaired receptor trafficking through the secretory pathway to the cell surface. As such, these intracellularly retained receptors may be amenable to 'rescue' using a pharmacological chaperone (PC)-based approach. PCs are small, cell permeant molecules hypothesised to interact with misfolded intracellularly retained proteins, stabilising their folding and promoting their trafficking through the secretory pathway. In support of the use of this approach as a viable therapeutic option, it has been observed that many rescued variant GPCRs retain at least a degree of functionality when 'rescued' to the cell

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surface. In this review, we examine the GPCR PC research landscape, focussing on the rescue of inactivating variant GPCRs with important roles in the HPG axis, and describe what is known regarding the mechanisms by which PCs restore trafficking and function. We also discuss some of the merits and obstacles associated with taking this approach forward into a clinical setting.

KEYWORDS
G protein-coupled receptor, HPG axis, pharmacological chaperone, pharmacoperone, reproduction

1 | INTRODUCTION

The human hypothalamic–pituitary–gonadal (HPG) axis is an endocrine signalling network responsible for gonadal development and maturation, gametogenesis and sex steroid production in males and females. Many of the hormones that co-ordinate the actions of this axis elicit their effects via members of the G protein-coupled receptor (GPCR) superfamily of cell surface signalling proteins. Many reproductive disorders have been attributed to genetic variants of these GPCRs, at all levels of the axis, resulting in hypogonadal phenotypes in both sexes. These variants frequently result in misfolding of the receptor protein, and subsequent intracellular retention as a result of detection by cellular quality control machinery. Treatment of hypogonadal patients relies on the administration of reproductive hormone analogues, with the regimen dependant on both the aetiology of the disorder and desired outcome. In many cases of hypogonadism, the desired outcome (such as restoration of fertility) is not achievable and there are often ramifications for the psychosocial well-being of the patients, many of whom suffer from social, sexual, emotional and self-esteem issues.[1] For those patients with inactivating GPCR variants for which current treatment options are not effective, alternative therapeutic approaches are necessary. One such approach is the use of pharmacological chaperones (PCs; also known as pharmacoperones) which represent a natural evolution to the concept of chemical chaperoning. Chemical chaperones bind non-specifically to misfolded variant proteins and stabilise their folding, preventing recognition by the cellular quality control machinery, thus restoring expression and activity. PCs in contrast have target specificity and are therefore valuable within a therapeutic context. Indeed, although there are currently no GPCR-targeting compounds licenced as PCs, there are Food and Drug Administration (FDA)-approved PC drugs targeting other protein families (such as migalastat and lumacaftor for the treatment of Fabry disease and cystic fibrosis [CF], respectively) currently in the clinic, validating this approach in the treatment of protein misfolding disorders.

The field has advanced considerably in the last decade, with many inactivating GPCR variants now successfully rescued with compounds displaying PC activity. Indeed, a major discovery is the significant diversity of molecules that have been described with observable chaperoning activity. These include both experimental and pre-clinical molecules as well as marketed therapeutics licenced for other purposes/properties. For example cinacalcet is an FDA-approved allosteric agonist of the calcium sensing receptor (CaSR) used to treat secondary hyperparathyroidism, that has recently been observed to act as a PC for intracellularly retained CaSR variants.[2] The field of PC research is evolving, with research endeavours now including a focus on understanding the mechanisms (both cellular and molecular) of PC-mediated rescue, and more recently, in the de novo design of bona fide PC agents with no intrinsic agonistic or antagonistic activity, in place of screening and repurposing of existing drugs and experimental compounds.

In this review, we discuss the identification of PCs targeting inactivating variants of GPCRs with important roles in the HPG axis, with a focus on inactivating variants of the neurokinin 3 receptor (NK3R), the Type 1 gonadotropin-releasing hormone (GnRH) receptor (GnRHR), the luteinising hormone (LH)/chorionic gonadotropin receptor (LHCGR, also known as LHR) and the follicle-stimulating hormone (FSH) receptor (FSHR). We describe the landscape of inactivating GPCR variants within the reproductive axis, and the rescue of these variants in terms of cellular localisation and restoration of function, in the context of the potential treatment of hypogonadism. We additionally discuss what is known regarding the mechanisms behind rescue of these variants, as well as the future of PC research and outstanding questions that need addressed to facilitate the translation of some of these exciting discoveries into tangible clinical applications.

2 | THE HPG AXIS AND THE ENDOCRINE CONTROL OF HUMAN REPRODUCTION

2.1 | The HPG axis

The HPG axis has a complex regulatory network with many inputs that regulate pulsatile release of GnRH from nerve terminals in the hypothalamic median eminence into the hypophyseal portal system.[3] These inputs include growth factors, neuropeptides, neurotransmitters and other hormones. Sex steroids, in particular, play a major role in imparting negative (and, in certain contexts, positive) feedback regulation of the axis. The discovery that GnRH neurons do not express oestrogen receptor-alpha[4] implied that an upstream input to the HPG axis network must exist to mediate the effects of sex steroids on GnRH secretion. In the early 2000s, genetic variants of a neuropeptide (kisspeptin) and its cognate receptor (KISS1R) expressed in neurons localised to the pre-optic area and infundibular nucleus of the

hypothalamus were determined to be causative in reproductive disorders including precocious, as well as delayed, puberty.[5–7] It has subsequently been demonstrated that kisspeptin stimulates the secretion of GnRH from GnRH neurons, and that kisspeptin neurons are responsible for co-ordinating and integrating various environmental, nutritional, metabolic, and endocrine signals that regulate GnRH secretion and downstream HPG axis activity (including feedback regulation by sex steroids).[8] In a subset of these kisspeptin neurons (termed KNDy neurons), localised in the infundibular nucleus, kisspeptin is co-expressed with the neuropeptide neurokinin B (NKB) and the opioid peptide, dynorphin A (DynA).[9]

NKB and DynA regulate kisspeptin secretion via autocrine/paracrine activation of NK3Rs and kappa-opioid receptors (KORs), respectively. A link between NKB and NK3R signalling in GnRH secretion was suggested through a number of genetic association studies in which NKB and NK3R variants were identified in hypogonadal patients.[10–14] Further evidence of a functional relationship was evidenced by the discovery that NKB co-localises with kisspeptin in the hypothalamus,[9,15] and that KNDy neurons express NK3R but GnRH neurons do not.[16] A number of pharmacological studies have subsequently provided information regarding the mechanism, with administration of NK3R antagonists and agonists inhibiting and stimulating LH secretion, respectively.[15–21] In addition, the administration of kisspeptin has been demonstrated to restore LH secretion in hypogonadal patients with NKB and NK3R inactivating variants,[13] and also in animal models treated with NK3R antagonists, suggesting that kisspeptin action is downstream of NKB signalling.[17,22]

In contrast to NKB, DynA has an inhibitory effect on kisspeptin secretion. Indeed, chronic opioid use has been associated with a hypogonadal phenotype[23] and morphine administration has been shown to cause a decrease in the biosynthesis of GnRH in rats—an effect abolished by co-treatment with the opioid antagonist, naloxone.[24] Several studies using animal models have also indicated that opioid receptors appear to mediate the negative feedback action of gonadal steroids on GnRH and LH pulsatile secretion (reviewed by Uenoyama et al.[25]). Furthermore, opioid antagonists have been shown to stimulate LH secretion and advance onset of puberty in a rat model,[26] restore gonadotropin pulsatility in women with hypothalamic amenorrhoea,[27] and restore normal cycles in polycystic ovarian syndrome,[28] thus providing evidence that opioid signalling is inhibitory in the context of the HPG axis. However, interestingly, to our knowledge, there are no studies describing KOR/DynA variants linked to reproductive phenotypes in humans.

Kisspeptin and NKB (and, to some extent, DynA) clearly play an integral role in the regulation of GnRH secretion (and thus the regulation and activity of the HPG axis). However, many other inputs and factors also play a role in the exquisitely orchestrated functioning of this neuroendocrine axis.

Binding of GnRH to GnRHRs on pituitary gonadotropes, drives the biosynthesis and release of the gonadotropins, LH and FSH, into the general circulation. These hormones bind to their cognate receptors (LHCGR and FSHR, respectively) expressed in the gonads. Activation of LHCGRs and FSHRs promotes germ cell production and maturation and sex steroid (oestrogens, androgens and progestogens) synthesis and secretion. In females, activation of LHCGRs on ovarian thecal cells drives the production and secretion of androgens, while FSHR activation in ovarian granulosa cells results in the induction of CYP19A1 (aromatase/oestrogen synthetase) expression which converts these androgens (androstenedione and testosterone) to oestradiol.[29] FSHR activation in granulosa cells also stimulates follicular growth. As the antral follicles develop they begin to express LHCGRs. Oocyte maturation and release from the dominant follicle is triggered by an ovulatory 'LH surge'. Following ovulation, the residual follicle forms the corpus luteum, where activation of LHCGRs by LH (or human chorionic gonadotropin [hCG] should pregnancy occur) stimulates secretion of progesterone that prepares the endometrium for implantation and placental development. In males, activation of FSHRs expressed on testicular Sertoli cells promotes spermatogenesis, while activation of LHCGRs on testicular Leydig cells results in the production and secretion of androgens (which are also are important for spermatogenesis[30]).

2.2 | Reproductive dysfunction resulting from genetic disruption of HPG axis activity

Functional disruption of the HPG axis results in a range of reproductive dysfunctions. Genetic variants that alter the function of gene products associated with the functioning, regulation or development of the axis, can result in gain-of-function phenotypes, such as precocious puberty, or loss of function phenotypes, characterised by compromised or absent pubertal development and impaired adult fertility.

GnRH deficiency (also known as hypogonadotropic hypogonadism [HH] or secondary hypogonadism) describes conditions in which there are defects in the production and/or action of GnRH, and is characterised by low gonadotropin levels and a subsequent impaired ability to produce sex steroid hormones. Cases where GnRH deficiency results from genetic abnormalities are referred to as congenital HH (CHH). Genetic aberrations that result in failure of GnRH neurons to migrate from the olfactory placode to the hypothalamus during embryonic development (Kallmann syndrome and other migratory failures), give rise to anosmic CHH,[31] while inactivating genetic variants of GnRH, GnRHR or upstream stimulators of the GnRH neuron (including KISS1 and NKB and their cognate receptors) result in normosmic CHH[5,7,10,32–34] since the GnRH and olfactory neurones migrate normally.

Inactivating genetic variants of HPG axis components downstream of the pituitary (such as the gonadotropin receptors) can also result in hypogonadism[35–87] (referred to as primary hypogonadism or hypergonadotropic hypogonadism). In these cases, sex steroid hormone levels are low but FSH and LH levels are elevated, due to reduced/absent negative feedback driving increased gonadotropin secretion via a functional GnRH system. In males inactivating LHCGR variants result in Leydig cell hypoplasia on a phenotypic spectrum, with milder forms presenting in the form of micropenis and/or hypospadias with suppression of fertility, and severe forms resulting in

infertile males presenting with female external genitalia. Females harbouring inactivating LHCGR variants exhibit amenorrhea/infertility but, in contrast to males, these patients undergo normal pubertal development, indicating that it is possible for pubertal development in females to occur against the backdrop of LH deficiency. Inactivating FSHR variants in females result in amenorrhea, ovarian failure, follicular arrest and infertility, while in males, inactivation of FSHR appears to result in a spectrum of phenotypes, from mild (reduced sperm quality, but retention of fertility), through to complete azoospermia.[88–91] Indeed, in one study, a single inactivating FSHR variant was identified in a male cohort that had variable degrees of fertility.[92] Congenital variants that affect the secretion and/or activity of gonadotropin hormones have also been identified and result in reproductive phenotypes.[88,93–105]

For secondary hypogonadism, replacement sex steroid therapy can successfully induce puberty and promote development of secondary sex characteristics while pulsatile GnRH or gonadotropin administration is a therapeutic option to restore gametogenesis.[106] However, for primary hypogonadism, although replacement sex steroid therapy can be used to promote sexual development, there are no effective options to restore gametogenesis/fertility in these patients.

3 | MAJOR GPCRS OF THE HPG AXIS

3.1 | G protein-coupled receptors

GPCRs are the largest family of cell surface receptors in humans, with approximately 800 members. Of these, approximately half modulate sensory functions (predominantly olfaction). The remaining ±400 are activated in response to endogenous signals. Given the large number of receptors and the broad spectrum of modulating ligands (cations, biogenic amines, peptides, steroids, etc.), it is unsurprising that they are responsible for regulating most physiological systems and are consequently associated with a plethora of pathologies and are important therapeutic targets. Indeed, there are currently over 700 FDA-approved GPCR drugs and over 300 more agents in clinical trials.[107,108]

GPCRs are characterised by a seven transmembrane domain (7-TMD) architecture, consisting of seven transmembrane helices (TMHs 1–7) connected by intracellular loops (ICLs 1–3) and extracellular loops (ECLs 1–3) and flanked by an extracellular N-terminus and intracellular C-terminus (although the latter is uniquely absent in mammalian GnRHRs[109]). The GPCR superfamily can be subdivided into five subfamilies (Glutamate-like, Rhodopsin-like, Adhesion, Frizzled/Taste and Secretin-like), based on sequence and structural homology.[110] By far the largest of these subfamilies (comprising over 80% of all GPCRs) is the Rhodopsin-like family, of which the KOR, KISS1R, NK3R, GnRHR, FSHR and LHCGR are all members. Intracellular signalling by GPCRs is mediated by interaction with their cognate ligands which induces a conformational change promoting their interaction with intracellular guanine nucleotide-binding proteins (G proteins), which, in turn, stimulate further downstream intracellular signalling cascades.

3.2 | GPCRs of the HPG axis

The KISS1R, KOR, NK3R and GnRHR interact with cognate peptide ligands and, like the majority of Rhodopsin-like GPCRs, have relatively short N-terminal regions. For these receptors, activation is generally elicited via direct hormone interaction with extracellular and 7-TMD regions close to the extracellular membrane surface. In contrast, the gonadotropin receptors (LHCGR and FSHR) have large N-terminal ectodomains (ECDs) of >300 amino acids comprising a series of leucine-rich repeats (LRRs) responsible for specific binding of their large dimeric glycoprotein hormone ligands (LH/hCG or FSH).[111] These ECDs are connected to the 7-TMDs by a 'hinge' region which contains a highly conserved 'intramolecular agonist'.[112,113] Interaction of hormone is believed to induce a conformational change that allows the hinge region to interact with, and activate, the 7-TMD, enabling G protein association.

4 | INACTIVATING VARIANTS OF HPG AXIS GPCRs

4.1 | Inactivating GPCR variants

Gene variants can comprise insertions, frameshifts, deletions, inversions and single-nucleotide variants (SNVs). These may have no effect (silent variants) or be pathogenic, by causing inactivating (loss-of-function) or activating (gain-of-function) phenotypes. Inactivating pathogenic GPCR variants can be further subclassified into five categories (Classes I–V) determined by the effect of the variant on receptor expression/function.[114] Class I variants arise from defects in receptor biosynthesis (including variants that alter receptor expression or cause premature truncation), Class II variants impede trafficking of GPCRs to the cell surface due to protein misfolding and abnormal conformational presentation, Class III variants have impaired ligand binding, Class IV variants have impaired receptor activation (and includes both variants unable to achieve an active conformation and those with impaired coupling to downstream signalling partners) and Class V variants are those identified/implicated in a particular pathophysiology but which have no known functional defects.[114]

A study examining thousands of disease-causing missense variants across a spectrum of protein families and pathophysiologies predicted that almost 30% resulted in protein instability/misfolding.[115] Indeed, misfolding/intracellular retention and failure to traffic to the cell surface due to disruption of intramolecular interactions (Class II defects) appears to be a major feature of variant GPCRs, and such variants have been implicated in numerous endocrine disorders.[114,116–119] This is perhaps not unexpected since most constituent amino acids are likely to be involved in intramolecular interactions that configure three-dimensional receptor structure (as opposed to the relatively few involved in ligand binding or interaction with intracellular signalling proteins) and thus their disruption will result in protein misfolding. For example, amino acid substitutions could disrupt TMH conformations through disruption of charged residue interactions, substitution with

helix breaking residues and steric disruption by substitution of amino acids with bulky side chains.

GPCR mRNAs are translated by ribosomes on the rough endoplasmic reticulum (ER). The emerging GPCR nascent protein enters the ER lumen, where it undergoes folding into its three-dimensional conformation assisted by ER-resident chaperone proteins and a suite of enzymes involved in glycan addition and post-translational modifications.[120] The mature protein then enters the secretory pathway for further post-translational modification and trafficking via the Golgi apparatus to emerge on the plasma membrane as a functional receptor. Receptor trafficking through the ER and Golgi to the cell surface following translation is regulated by an ER quality control system (QCS). The QCS examines newly synthesised proteins to ensure that only correctly assembled and folded proteins are trafficked through the secretory pathway to the cell surface. The presence of unpaired cysteines, exposed hydrophobic residues and other markers of misfolding result in retention of the proteins in the ER and activation of the unfolded protein response.[121] This process involves upregulation of ER protein folding machinery and downregulation of global translation to allow rectification of the misfolding and prevention of an accumulation of misfolded proteins. Should the misfolding persist (terminal misfolding), degradative pathways are activated, whereby the misfolded proteins are retro-translocated to the cytosol and ubiquitinated prior to degradation by the proteasome via a process termed ER-associated degradation.[122] Thus, misfolded GPCRs are retained intracellularly and/or degraded rather than being trafficked to the cell surface to be available to interact with their cognate ligands.

Although decreased cell surface expression is a common outcome of variants located within GPCR protein-coding regions, it is interesting to note that there are examples where variants have enhanced cell surface expression. One such example is the LHCGR variant insLQ, where an insertion results in a more efficient signal peptide (a signal sequence that facilitiates targeting to the secretory pathway), and an increase in cell surface expression (and thus activity) of the receptor.[123]

4.2 | Inactivating GnRHR variants

Interestingly, with the exception of the rat and mouse receptors, wild-type (WT) mammalian GnRHRs are poorly expressed at the cell surface, and only a small proportion (variably estimated to be between 1% and 50%) of total cellular GnRHR is located at the plasma membrane.[124,125] The reasons for this have not been fully elucidated, but it has been suggested that, as the complexity of the reproductive process among species increases, there is a concomitant decrease in the proportion of GnRHR observed at the plasma membrane.[126] It is unclear why this mechanism has been selected from an evolutionary standpoint, although it is thought that the high concentration of ER-localised GnRHR may provide a readily available 'pool' of receptor negating the requirement for de novo synthesis, thus affording a sensitive means to rapidly alter GnRH responsiveness.

Over 40 inactivating variants of the human GnRHR have been attributed to a CHH phenotype, with greater than 50% of those studied exhibiting an increase in intracellular receptor retention and a decrease in plasma membrane expression (Class II variants).[127,128] An even greater number of GnRHR variants have been characterised in vitro (encompassing both naturally occurring and lab-generated variants) and, through these studies, a number of intra-molecular interactions have been identified as important for GnRHR-folding and ER trafficking (in addition to those important for hormone binding and receptor signalling functionalities). These studies highlight the role that structural motifs play in the stability and trafficking of the GnRHR (and GPCRs in general) and the dramatic effects that single variants can have on receptor localisation and activity. For example, rat and mouse GnRHRs exhibit a deletion of residue 191 (located in ECL 2) that is present in other mammalian GnRHRs (primate receptors have a lysine [K] located at this position, while many other mammalian GnRHRs have glycine [G] or glutamic acid [E]). Deletion of K191 of the human GnRHR (ΔK191) promotes a substantial increase in cell surface expression.[126,129] From a mechanistic standpoint, this amino acid appears to interfere with disulphide (cysteine; C) bridge formation between the receptor N-terminus and ECL2 (C14–C200) that is required for efficient cell surface trafficking of human (but not rat or mouse) GnRHR.[129] Molecular dynamics simulations suggest that deletion of K191 results in a shorter and more stable distance between the sulphur atoms of C14 and C200, promoting a trafficking competent receptor structure.[130] The distance between these residues appears to be the important factor, rather than the bridge itself, as substitution of C14 in the human GnRHR with a serine that disrupts the formation of the disulphide bridge results in signalling activity comparable to WT in the presence of the ΔK191 variant. In this case, the distance between positions C14 and C200 is predicted to be short due to an extracellular hydrogen-bond network formed by the introduced serine moiety.[130] Another example is a naturally occurring variant, E90K located in TM2, which was first identified in siblings with CHH.[131] E90 appears to form important intrahelical interactions, with disruption of these interactions destabilising the receptor, resulting in retention and a loss of hormone responsiveness.[125,132–137]

4.3 | Inactivating LHCGR variants

At least 34 naturally occurring inactivating variants of the LHCGR have been described in patients suffering from reproductive dysfunction (primary hypogonadism).[35–61] Extensive analyses of rodent LHCGRs by Segaloff et al. and others, in which the importance of various regions/features for receptor function have been examined, have indicated that impaired cell surface trafficking is a common outcome of the introduction of variants into this receptor.[138] Furthermore, examination of cell surface localisation of 21 SNVs or in-frame deletions of the human LHCGR has indicated that impaired cell surface trafficking is the most common defect (Class II variants). The majority (90%) resulted in reduced cell surface expression, of which 71% had severely impaired cell surface expression (<25% of WT receptor

levels).[39,61,117] These Class II variants are located within, and thus affect all, regions of the LHCGR protein (ECD, hinge region and 7-TMD).

The phenotypes of patients harbouring LHCGR variants with impaired cell surface expression range from mild (e.g. variant S616Y for which 46,XY individuals presented with a micropenis and primary hypogonadism)[41,44] to severe (e.g. variant A593P for which genetically XY individuals presented with female external genitalia).[57] In general, the severity of patient phenotypes correlates with degree of retention of the variant receptors. For example, while the A593P variant has been shown to have little/no measurable cell surface expression or presence of mature receptor species, the S616Y variant LHCGR displays some cell surface expression and presence of a small amount of receptor in a 'mature' form.[139,140] The observable phenotypes resulting from homozygous and compound heterozygous inactivating variants of LHCGR follow an autosomal recessive pattern of inheritance. However, it is also worthy of note that the LHCGR is known to dimerise,[141] with possible ramifications for identified heterozygous LHCGR variants. Indeed, inactivating LHCGR variants have been demonstrated to impact cell surface expression and possibly function of WT LHCGR, in a number of in vitro studies, via a dominant negative mechanism.[142,143]

4.4 | Inactivating FSHR variants

Compared to the closely related LHCGR, fewer naturally occurring pathogenic FSHR variants have been identified.[89] Nonetheless, at least 21 naturally occurring inactivating or partially inactivating FSHR variants have been described and have been linked to primary hypogonadism.[62–87] Similar to the LHCGR, examination of the cell surface expression of a selection of missense variants has indicated that the majority (85%) cause reduced cell surface expression (Class II variants), with (70%) having severely impaired cell surface expression. Again, similar to the LHCGR, the Class II variants are dispersed throughout the different receptor domains.

4.5 | Inactivating NK3R, KISS1R and KOR variants

Several pathogenic inactivating NK3R variants have also been identified, many of which have been linked to reproductive dysfunction (CHH).[10,11,14,144–146] A recent study examining seven missense coding region variants indicated that, similar to the GnRHR, LHCGR and FSHR, the majority (71%) are Class II and result in severely reduced cell surface expression of <25% of WT levels.[147]

To date, a number of loss-of-function KISS1R variants have been described in patents with CHH.[5,7,148–164] However, it is unclear, for many of these variants, whether loss of cell surface expression contributes to the loss-of-function phenotype due to a lack data describing the localisation of the variants. Of those for which cell surface localisation has been examined, one variant (L148S) was found to have no effect on localisation and had similar cell surface expression levels as the WT receptor in vitro.[5,148] Conversely, a homozygous variant (F272S) identified in six related individuals with CHH, was demonstrated to have significantly reduced cell surface expression in fluorescent microscopy experiments.[164]

As discussed above (Section 2.1), KOR variants have not been associated with a reproductive phenotype in humans. However, they have been implicated in increased stress and drug-relapse risks,[165,166] although the consequences of these variants in terms of receptor expression/functionality are largely unknown. Interestingly, like the GnRHR, only a proportion (approx. 75%) of WT KORs are expressed at the plasma membrane in the mature form, with the remainder being intracellularly retained in an immature form.[167]

5 | PHARMACOLOGICAL CHAPERONE RESCUE OF HPG AXIS GPCRs

5.1 | Pharmacological chaperones

Although many GPCR-targeting therapeutics exist, these predominantly act as agonists (which stimulate receptors) or antagonists (which inhibit receptor activation by endogenous agonists).[108] In these cases, activity relies on the availability and accessibility of functional target receptors.

The term PCs has been coined to describe cell-permeant small-molecule agonists and antagonists that can enter cells and engage with nascent proteins to stabilise their folding and thus promote their expression and intracellular trafficking. In contrast to chemical chaperones that act non-specifically to aid protein folding and/or prevent aggregation,[168] PCs have an inherent target specificity.[169] Their binding is thought to aid in stabilising three-dimensional protein conformation of misfolded variant (or otherwise inherently unstable) receptor proteins by acting as molecular scaffolds, thus preventing detection and retention/degradation of misfolded proteins by the QCS and aiding their correct processing and trafficking through the secretory pathway.

In the case of GPCRs, PC treatment results in 'rescued' cell surface receptor expression and localisation, restoring their availability for interaction with exogenous/endogenous ligands. Importantly, many intracellularly retained Class II variant GPCRs, have been found to maintain at least a degree of their intrinsic functionality (hormone binding and signal transduction capacity) following PC rescue.[170] Thus, there is potential for partial or complete rescue of function of these misfolded receptors should their cell surface expression be restored.

The development of PC therapeutics is still a relatively embryonic field. However, PCs have been shown to be effective in the treatment of various disorders caused by intracellularly retained proteins. Indeed, PCs are currently in clinical use for several disorders in which misfolded proteins of other (non-GPCR) families are implicated, including CF, Fabry disease and phenylketonuria.[171–177] Although no PCs targeting GPCRs are currently utilised therapeutically, a number have been shown to enhance variant, and in some cases WT, GPCR

cell surface expression (reviewed by Tao and Conn[170] and others). Several studies have also translated the in vitro rescue of variant GPCR function to in vivo studies. One such study examined variants of the melanocortin 4 receptor (MC4R), a central regulator of energy homeostasis, associated with familial obesity.[178] It was demonstrated that administration of a PC, UM0130866, could successfully restore the anorexigenic effect of an MC4R agonist, melanotan II, in a humanised mouse model harbouring a R165W MC4R variant, thus establishing that treatment with this PC is able to partially reverse the effects of MC4R deficiency.[178] Another study utilised a transgenic mouse model of autosomal dominant retinitis pigmentosa (a major cause of vision loss), harbouring a T17M variant that results in intracellularly retained rhodopsin. Administration of vitamin A or an orally active synthetic retinal analogue, SRD005825, was found to delay retinal degeneration in this model.[179,180] Transgenic *Xenopus laevis* models of retinitis pigmentosa harbouring another rhodopsin variant, P23H, have also demonstrated increased receptor trafficking by 11-cis retinal.[181] In the context of the HPG axis GPCRs, it has been demonstrated that the hypogonadal phenotype of knock-in transgenic male mice harbouring an intracellularly retained E90K variant of the GnRHR could be partially rescued by treatment with a small-molecule GnRHR antagonist, IN3[182] (see Section 5.2.1 for more details). Further to these animal studies, GPCR PC activity has also been demonstrated in humans. Inactivating variants of renal arginine-vasopressin V_2 receptor (V2R) result in congenital nephrogenic diabetes insipidus (NDI) and a small-molecule compound (Relcovaptan; SR49059) has been shown to decrease urine volume and increase urine osmolality by 50% in patients harbouring NDI-associated retained variant V2Rs (R137H, W164S and Del G185-W193).[183]

5.2 | Pharmacological chaperones for HPG axis GPCRs

As described previously (Section 4), naturally-occuring NK3R, GnRHR, LHCGR and FSHR SNVs and/or deletion variants have been shown to predominantly result in loss of receptor cell surface expression, making them good candidates for PC rescue. Indeed, PCs have been described for all four of these receptors and also for the KOR. Although no KISS1R-directed PCs have been reported thus far, small-molecule KISS1R-selective analogues have been developed[184,185] so there is scope for future investigation into their potential as PCs for Class II KISS1R variants.

Due to their vital roles in reproduction, HPG axis GPCRs are important therapeutic targets. Hormones or analogues produced through modification of the native hormones to improve their pharmacological parameters (half-life, stability, etc.) or to confer different pharmacological properties (agonists vs antagonists etc.) are commonly utilised. However, as small-molecule (non-peptide) agents have potential benefits with respect to administration, patient convenience, preparation consistency, stability and dosing, there has been, and continues to be, much effort made to develop such compounds, providing a large repository of potential cell-permeant (orally active) small-molecule compounds for application in PC rescue studies. In theory any cell-permeant ligand that can interact with a GPCR will stabilise its conformation to some extent and therefore have the potential to act as a PC for destabilising variants of that receptor. Indeed, 'repurposing' or 'repositioning' of existing small molecules targeting GPCRs has provided the vast majority of the identified PCs.

5.2.1 | GnRHR pharmacological chaperones

The human GnRHR can perhaps be considered paradigmatic in the study of inactivating GPCR variants and their rescue by PCs. Indeed, much of the knowledge regarding the action of GPCR PCs has been derived from an extensive body of work studying variants of this receptor. There are also a substantial number of small-molecule modulators of the GnRHR in development due to the importance of GnRHR signalling at the level of the gonadotrope and indirectly in hormone-dependent diseases. GnRH analogues are administered therapeutically in several settings, including assisted reproduction protocols, as well as for the inhibition of sex steroid secretion for the treatment of hormone-dependent disease such as prostatic cancer (androgen-deprivation therapy) and endometriosis. Many different chemical classes of compounds have been developed as non-peptide GnRH antagonists (reviewed by Heitman and Ijzerman[186]), although, until very recently,[187] no non-peptide GnRHR agonists had been developed.

A number of Class II naturally occurring and lab-generated GnRHR variants have been shown to be amenable to PC rescue. The WT human GnRHR has also been demonstrated to be receptive to PC activity with substantial increases in plasma membrane expression (and signalling) observed following treatment with various small-molecule antagonists.[129,133,135] Notably, WT rat and mouse GnRHR do not display increased plasma membrane expression in response to PC treatment, likely due to their inherently increased stability (due to the lack of a K191 residue) and resultant increased trafficking efficiency.[129] One of the first examples of PC rescue of inactivating variants of the GnRHR was a study looking at five naturally occurring Class II variants implicated in CHH. In response to treatment with a small-molecule indole antagonist, IN3, all five intracellularly retained variants showed an increase in radiolabelled GnRHR agonist ([^{125}I]-Buserelin) binding.[134] Subsequently, a large number of Class II GnRHR variants (naturally-occurring, and lab-generated) have been shown to be 'rescuable', and several antagonists derived from four non-peptide chemical subclasses (indoles, quinolones, erythromycin macrolides and pyrimidinones) have demonstrated PC activity for variant and WT GnRHRs variant.[133,188–191] Remarkably, of the Class II GnRHR variants treated with PCs, approximately three quarters demonstrate observable rescue to the cell surface.[133,188,192,193]

It is important to note that rescue of cell surface expression of variant receptors does not assure rescue of function, as this would assume that the variants do not impair binding/signalling capability of the receptor in addition to affecting their trafficking. However, in many cases, rescue of function (signalling response), in addition to cell

surface localisation, of rescued GnRHR variants has been demonstrated. Indeed, functional rescue of a GnRHR variant by a PC has been demonstrated in vivo. The Class II E90K variant was introduced into mice by homologous recombination, with the male mice homozygous for E90K displaying reduced testes size but retaining fertility, while female homozygotes generated antral follicles, but did not ovulate.[182] Interestingly, the targeting strategy left a loxP-flanked neomycin resistance gene expression cassette in intron 1, and when this was not removed (E90Kneo) the phenotypes were more severe, due to effects of the cassette on transciption of the gene encoding GnRHR.[182] The males homozygous for E90Kneo had reduced testes weight and infertility, while the females were infertile with no follicular development past the secondary follicle stage. When the PC antagonist, IN3, was administered in a pulsatile manner for 30 days to E90Kneo male homozygotes, testes morphology and a number of metrics of testes function were improved including increased testes weight, increased seminiferous tubule diameter and the presence of elongated spermatids[182] (Figure 1). Sperm morphology also began to approximate that of WT mice, with less apparent defects, and increased motility.[182] Sperm isolated from the IN3-infused animals was successfully used in an in vitro fertilisation protocol and resulted in a live birth. On a biochemical level, IN3 infusion also increased the expression of steroidogenic markers in the testes.[182]

Interestingly, as mentioned above, individual inactivating GnRHR variants can be rescued by a spectrum of PCs from different chemical classes. While the exact mechanisms have yet to be elucidated, it is thought that this is due, at least in part, to the small-molecule GnRHR modulators having similar modes of receptor interaction. For example, it has been demonstrated that the indole and quinolone classes of PC dock in a similar binding pocket, facilitating stabilisation of TM2/TM3 interactions by utilising an acidic residue, D98, in TM2 as an anchor point.[194] Therefore, compounds from these different classes appear to orientate in a similar manner within the 7-TMD and form several conserved interactions, thus stabilising a trafficking-competent structure, and providing a mechanism by which multiple structurally distinct PCs could rescue a single variant. Indeed, structure–function analyses have revealed that the binding sites of small-molecule GnRHR-interactive compounds are similar and partially overlap with the orthosteric-binding site of GnRH within the 7-TMD bundle.[195–197]

5.2.2 | LHCGR pharmacological chaperones

Gonadotropins are commonly administered exogenously to females undergoing assisted reproductive therapy, whereby folliculogenesis is induced by daily administration of FSH and, upon follicle maturation, ovulation is stimulated by the administration of LH/hCG for oocyte harvesting. There have been significant advances in developing non-peptide gonadotropin therapeutics and a number of compounds comprising a range of structural scaffolds have been developed targeting the LHCGR (reviewed in 198,199), although none have yet made it into clinical use.

The most advanced series of LHCGR-targeting small-molecule agonists to be developed are the thienopyrimidines. One of these, LHR-Chap (also known as Org42599 or as Org43553 in its hydrochloride salt form) has high affinity for the LHCGR[200] and has also been shown to act as an LHCGR PC able to restore cell surface expression (and function) to several Class II LHCGR variants[140,201] (Figure 2). Interestingly, like the majority of small-molecule gonadotropin analogues identified thus far, LHR-Chap is an 'allosteric' agonist, referring to the fact that they interact with the receptors at an allosteric

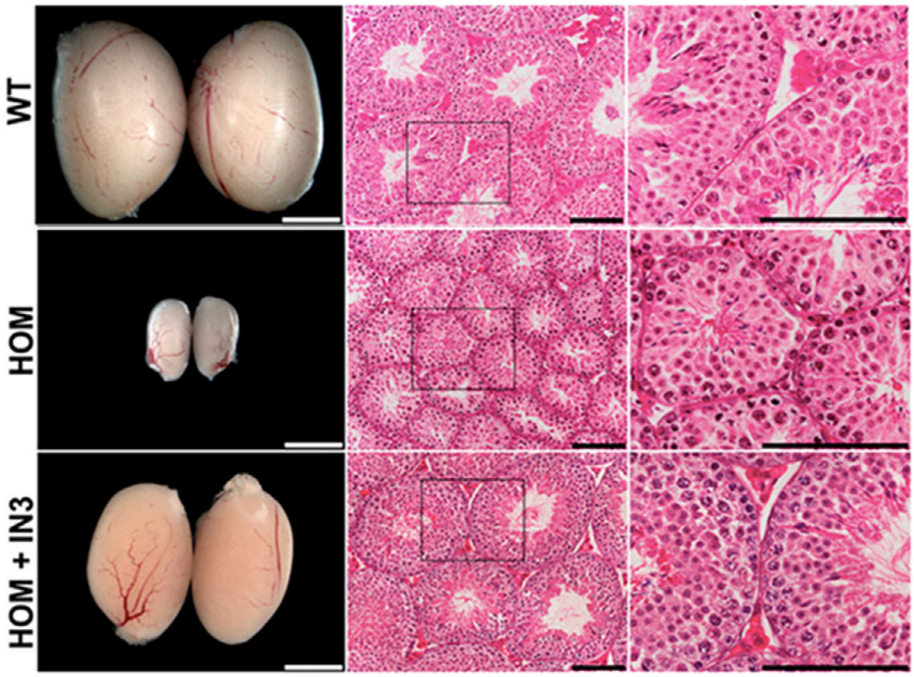

FIGURE 1 Pharmacological chaperone rescue in an in vivo mouse model of hypogonadotropic hypogonadism. Mouse testes from wild-type (WT) or homozygous E90Kneo/E90Kneo mice (HOM) were imaged with a stereomicroscope, and histology was examined by haematoxylin and eosin staining. The testes of HOM males were smaller in size compared to WT and contained few to no eosin-stained Leydig cells or elongated spermatids. Treatment of HOM males with the pharmacological chaperone IN3 for 30 days (HOM + IN3) resulted in increased testis size, sperm concentration and various metrics of sperm morphology and viability. Reproduced with permission from Janovick et al.[182]

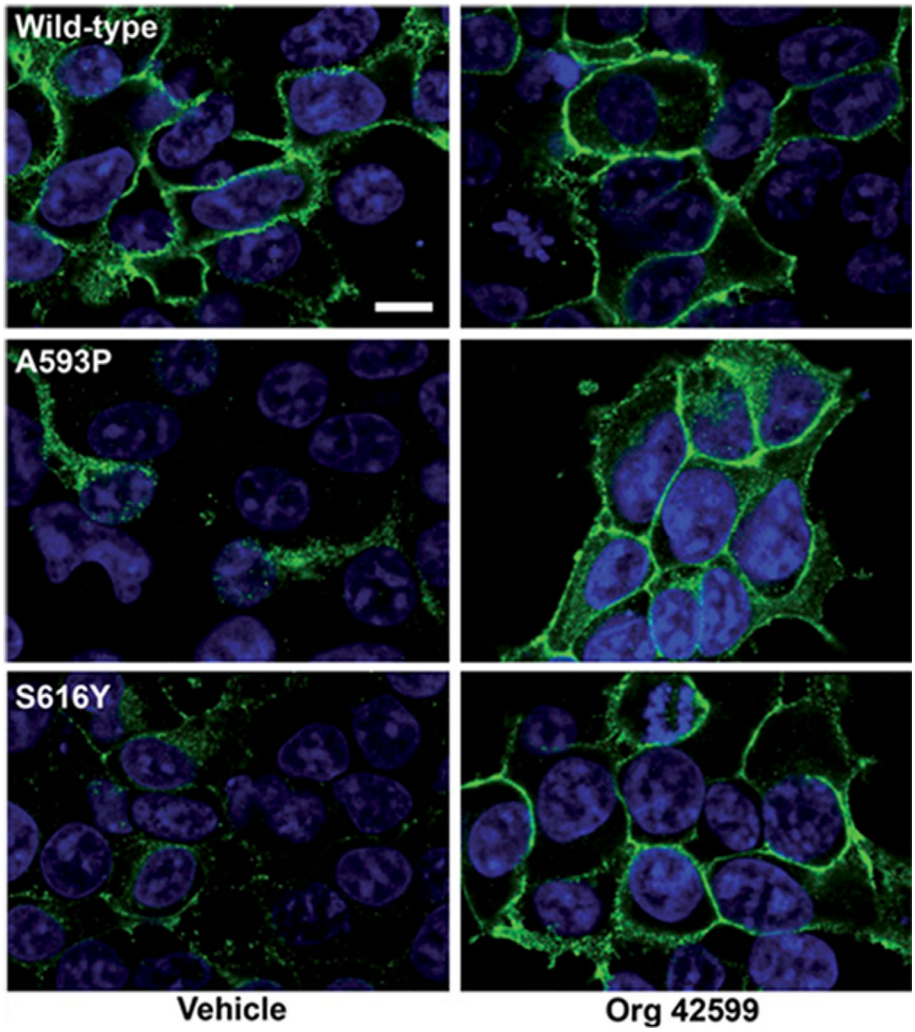

FIGURE 2 Cell surface expression of A593P and S616Y variant LHCGRs is altered after incubation with LHR-Chap. Cells expressing wild-type, A593P or S616Y variant LHCGRs were incubated in the presence of vehicle (left column) or 1 μM LHR-Chap (Org 42599); right column) for 24 h before fixation, fluorescent labelling, and confocal imaging. LHCGRs (myc-tagged) are labelled in green and cell nuclei in blue. Scale bar: 10 μm. Reproduced with permission from Newton et al.[140] LHCGR, luteinizing hormone/choriogonadotropin receptor, also known as LHR.

('other') site within the 7-TMD of the receptor distinct from the ECD-located orthosteric binding site of the native gonadotropin hormones.

Examination of 14 Class II LHCGR variants with severely reduced cell surface expression has indicated that treatment with LHR-Chap can significantly increase cell surface expression of 5 (36%).[61,140,201] The cell surface expression of the variant LHCGRs was increased to varying degrees by LHR-Chap, with some demonstrating no response, some only marginal increases (to 13%–17% of WT), and others much larger increases (to 53%–68% of WT), as measured by number of hormone-binding sites on intact cells.[201] Rescue of cell surface expression of LHCGR variants by LHR-Chap has also been shown to be time and dose-dependent, with maximal effects seen after 24 h of incubation with 1–10 μM of the PC.[140] In addition to increasing cell surface expression, total cellular levels of LHCGR variant receptor expression and the proportion that presented with 'mature' post-translational glycosylation was also increased upon PC treatment.[140]

It is interesting that, in comparison to the GnRHR variants, the cell surface expression of a smaller proportion of LHCGR variants appear to be rescued by PC treatment. However, unlike the GnRHR, the LHCGR (and FSHR) contains a large ECD region distinct from their 7-TMD. As mentioned above (Section 4.3), Class II LHCGR variants are distributed throughout the LHCGR structure, in both the ECD, hinge and 7-TMD regions. While none of the ECD-located variants could be rescued, the majority (63%) of 7-TMD variants demonstrated increased cell surface expression following LHR-Chap incubation. LHR-Chap interacts within an allosteric binding pocket located within the 7-TMD comprising residues from TM3, TM5, TM6 and TM7, ECL2, ECL3 and the hinge region[202] (Figure 3); therefore, it is not surprising that its binding is able to stabilise variants located in the 7-TMD but not the ECD region (either by directly interacting with the variant residues or indirectly stabilising delocalised TMHs that harbour the variants).

In addition to increased cell surface trafficking, an increase in functionality (hormone-induced signalling) has been demonstrated for many of the rescued LHCGR variants. Indeed, following pre-incubation of cells with LHR-Chap, hCG-induced signalling was significantly increased for the majority (3/5; 60%) of rescued variants.[201] However, it is important to note that, although hormone-induced responses were increased, these were lower than would be expected based on LHR-Chap effects on cell surface expression, presumably due to some perturbation of the signal transduction capabilities of these variant receptors. Despite the curtailed signalling responses

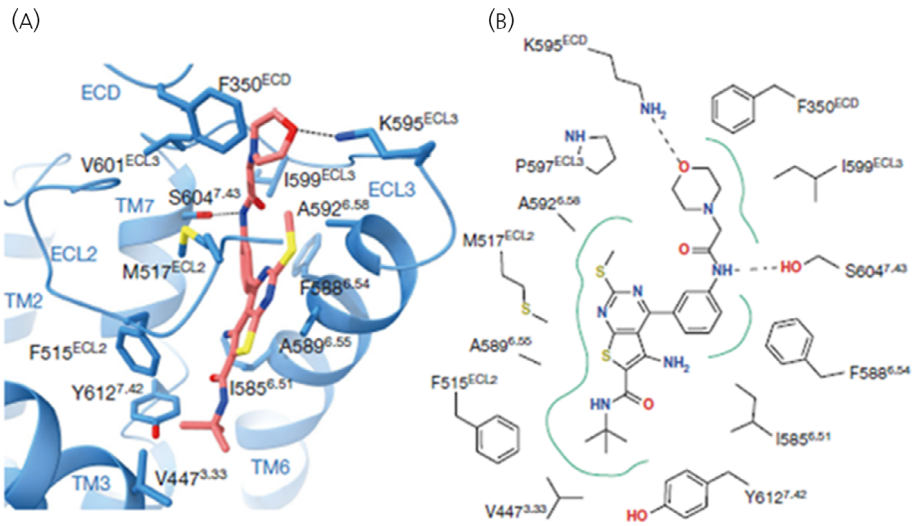

FIGURE 3 Cryo-electron microscopy derived structures of LHR-Chap docked to LHCGR. A. Structural interactions and B, Schematic representation of LHR-Chap–LHCGR interactions within the LHCGR 7-TMD. Hydrogen bonds are shown as black dashed lines and hydrophobic interactions are shown in green. Amino acid residues are numbered using the Ballesteros–Weinstein system.[233] Reproduced with permission from Duan et al.[202] LHCGR, luteinizing hormone/choriogonadotropin receptor, also known as LHR.

of the rescued variants, these data provide a proof of principle for the functional rescue of pathogenic LHCGR variants with PCs and it will be interesting to see if/how these in vitro findings translate into animal or clinical studies. LHR-Chap (and a related thioenopyrimidine Org 43902; also known as Org 41841) have already successfully navigated Phase I toxicology and safety studies and have shown in vivo activity (in inducing ovulation in healthy female volunteers)[203] further supporting their potential therapeutic development as PCs.

Due to the high degree of similarity between the gonadotropin hormone receptors, a potential concern regarding the use of LHR-Chap as a PC therapeutic may be its lack of selectivity. LHR-Chap is also able to activate the FSHR, at an approximately 10-fold lower potency than observed at the LHCGR.[200] Thus, treatment may result in concomitant nonspecific activation and possible densensitisation of FSHRs. One possible means to circumvent this issue could be the use of bivalent ligands whereby compounds with different properties are conjugated to produce a single compound that selectively activates one receptor while antagonising another. Production of such compounds targeting gonadotropin receptors have been explored with varying degrees of success.[204]

Unlike the GnRHR, cell surface expression of the WT LHCGR was not increased by PC treatment.[140,201] This is not unexpected as the WT human GnRHR is expressed at low levels at the cell surface relative to the total cellular pool, while the WT human LHCGR is expressed predominantly at the cell surface in a mature form when expressed endogenously or exogenously.[142,205]

5.2.3 | FSHR pharmacological chaperones

Based on its agonist activity at the FSHR and effectiveness as an LHCGR PC, the ability of LHR-Chap to rescue cell surface expression of a range of naturally occurring and laboratory-generated Class II FSHR variants has been examined. These data have indicated that LHR-Chap is also able to act as an FSHR PC, with the potency of rescue being proportional to the potency of activation at both of the gonadotropin receptors (our unpublished data). Likewise, a related thienopyrimidine LHCGR agonist, Org 41841, has also been proposed to act as an FSHR PC[206] although, in this case, the functional effect was limited, and only one variant (of several tested) displayed rescue. In a therapeutic setting, rescue of variant FSHRs with an LHCGR agonist would be impractical due to the concomitant nonspecific LHCGR activation and possible desensitisation. Therefore, attempts have also been made to identify FSHR-selective PCs.

Like LH/hCG, FSH is used clinically for assisted reproductive therapies and there have been substantial efforts to identify small-molecule FSH-selective analogues. The potential use of orally active FSHR analogues as novel oral contraceptives, with greater specificity and reduced risk of side effects, compared to current steroidal contraceptive drugs has further fuelled these efforts. Compounds of different chemical scaffolds have been identified as FSHR-selective ligands but despite several promising lead compounds emerging,[207] none have yet made it into clinical use.

When a selection of FSHR selective small-molecule compounds from different chemical classes were tested for PC activity, an increase in cell surface expression of 6 of 11 Class II FSHR variants was observed. Of the compounds tested, a dihydrobenzoindazole agonist, CAN 1404 (originally described in the published patent WO2011/012674[208] and which has 400-fold greater potency at the FSHR than the LHCGR[209]) was most effective. Like LHR-Chap, CAN 1404 binds allosterically within the 7-TMD of the FSHR and thus, unsurprisingly, only variants located in the 7-TMD were rescued. Of the variants tested, seven were located in the 7-TMD and the cell surface expression of 86% (6/7) of these was significantly increased (to 57%–89% of WT levels) following CAN 1404 incubation.[209] Importantly, a concurrent increase in FSH-induced responsiveness was also observed for 67% (4/6) of the rescued variants.[209]

As seen with the LHCGR, no increase in WT FSHR expression was observed upon PC (CAN 1404) treatment (again, likely reflecting the high efficiency of WT receptor trafficking). Interestingly, contrary to these observations, when treated with Org 41841, an increase in the number of FSH binding sites present on cells expressing WT FSHR

was observed. In the same study, Org 41841 treatment was also found to increase FSH-induced responses in cells expressing the ECD-located Class II A189V variant FSHR.[206] Not discounting factors relating to differences in cell lines or methodologies utilised in these studies, these observations are somewhat surprising as the A189V variant is located within the FSHR ECD distant from the 7-TMD allosteric binding site. Furthermore, Org 41841 is only able to induce FSHR activation at very high (millimolar) concentrations,[210] but the increases in WT and A198V variant receptor binding sites were observed at much lower (micromolar) concentrations.

5.2.4 | NK3R pharmacological chaperones

In addition to its important roles within the HPG axis, the NK3R is a pleiotropic receptor associated with a number of (patho)physiological processes, including hot flushing in post-menopausal women, mood disorders, chronic pain, and neurodegenerative disorders.[211,212] Consequently, there has been considerable endeavour in developing NKB peptide and small-molecule analogues for central nervous system indications (e.g. anxiety, schizophrenia and pain), gastrointestinal tract indications (e.g. irritable bowel syndrome) or menopausal vasomotor symptoms (hot flushes). Small-molecule NK3R-targeting compounds fall into two main structural classes: dichlorophenylalkylpiperidines and quinolones. Such small-molecule modulators of NK3R activity have been shown to utilise a binding pocket deep within the 7-TMD bundle that is distinct from, but overlaps with, the NKB interaction site.[213]

Two quinolone antagonists, talnetant (also known as SB 223412[214]) and M8 (also known as 8m[215]), have been shown to have PC activity and M8 was able to significantly increase cell surface expression of 80% (4/5) of a selection of severely retained Class II NK3R variants to levels similar to that of the WT NK3R.[147] As with the LHCGR, rescue of the NK3R variants to the cell surface was concurrent with an increase in the mature glycosylation status of the receptors.[147] Furthermore, all the M8-rescued variant receptors displayed robust increases in hormone responsiveness (Figure 4).[147] Like the gonadotropin hormone receptors, no effect on cell surface expression of WT NK3R was observed following PC treatment.[147]

5.2.5 | Kappa opioid receptor pharmacological chaperones

A large number of peptidic and small-molecule opiate-like compounds with KOR activity have been developed, many of which have been licenced for clinical use. KOR-selective agonists have primarily been developed as analgesics and KOR antagonists for use in the treatment of opioid overdose and alcohol dependence.[216] Thus, there is a spectrum of small-molecule compounds available with potential KOR PC activity. Although rescue of cell surface expression of KOR variants has yet to be demonstrated, several compounds have been tested for PC activity at the WT KOR. One such non-peptide non-selective opioid receptor antagonist, naloxone, has been shown to enhance receptor maturation status in vitro, resulting in increased KOR trafficking and cell surface expression.[217] Furthermore, variants of other members of the opioid receptor family have been shown to be responsive to PCs. For example, the δ-opioid (DOR)-selective antagonist/weak partial agonist naltrexone has been found to act as a PC able to promote the maturation and ER export of both WT DOR and an intracellularly retained DOR variant, D95A, increasing their

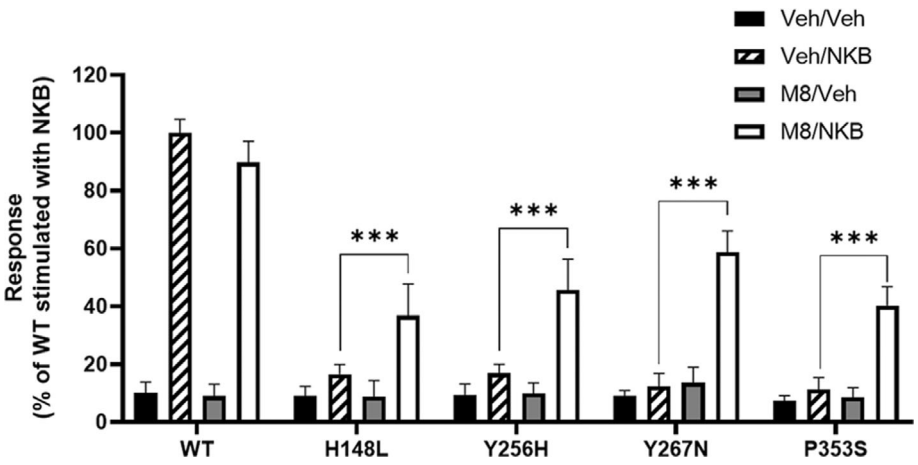

FIGURE 4 M8-mediated trafficking of intracellularly retained inactivating NK3R variants to the cell membrane restores neurokinin B-mediated stimulation. Receptor signalling was measured using an inositol phosphate accumulation assay. HEK 293-T cells expressing wild-type (WT) NK3Rs or intracellularly retained inactivating NK3R variants were pre-treated in the absence (Veh; black and striped bars) or presence (M8; grey and white bars) of 1 μM M8 for 24 h. Following washing, cells were then incubated for one hour in the absence (Veh; black and grey bars) or presence (NKB; striped and white bars) of 100 nM neurokinin B. Data are expressed as percentage of WT NK3R in the absence of M8 and stimulated with NKB ***$p < .001$, Student's t-test for comparison of NKB response with/without M8 pre-treatment. Modified and reproduced with permission from Anderson et al.[147] NKB, neurokinin B; NK3R, neurokinin 3 receptor.

expression at the cell surface.[218] Thus, it is predicted that KOR variants will also be responsive to PC treatment.

5.3 | Mechanistic observations of pharmacological chaperone action

While the PC studies described above have identified a large number of variants of different HPG axis GPCRs amenable to PC rescue, they have also served to highlight some fundamental principles underlying reduced cell surface expression of variant GPCRs and the ability of PCs to 'rescue' GPCR variants.

Visualisation of the cellular localisation of variants with poor cell surface expression has confirmed that their reduced cell surface expression is due to intracellular retention of the receptors (as opposed to other factors such as increased turnover or internalisation at the cell surface)[140,147,209] and that they co-localise with fluorescently labelled ER markers.[201] Western blotting analyses of cells transfected with WT and variant receptors has also demonstrated that the predominant forms of variants with poor cell surface expression are endoglycosidase-sensitive, indicative of the receptors remaining in an 'immature' state (prior to ER exit/further processing within the secretory pathway).[139,147] Furthermore, total receptor content of cells expressing intracellularly retained receptors was not reduced, or was reduced only marginally, compared to cell surface expression,[117,147] indicating that, although in some cases reduced receptor synthesis/increased degradation may be playing a role, the major contributor to poor cell surface expression of the variant receptors is a failure to traffic to the cell surface.

Pre-treatment of cells expressing variant GnRHRs[219] or LHCGRs (our unpublished data) with cycloheximide (to inhibit de novo protein synthesis) does not affect the ability of PCs to increase receptor cell surface expression. This suggests that PCs act post-translationally to rescue a pool of previously synthesised misfolded receptors retained within the ER, thereby increasing the trafficking of receptor protein to the cell surface and reducing the amount of receptor targeted to degradation pathways. From studies examining LHR-Chap rescue of LHCGR variants, it has also been shown that washing out LHR-Chap does not affect rescue of cell surface localisation of the variants in the short term,[201] indicating that the PC interaction is only required for successful trafficking from the ER and not for maintaining cell surface expression following plasma membrane insertion. However, over a longer time period (18 h) removal of the PC led to a reduction in cell surface expression of 'rescued' variant LHCGRs back to pre-treatment levels,[140] likely reflecting turnover of the receptor protein at the cell surface, and a failure to replenish from the ER. Thus, depending on the turnover rate of the specific receptor of interest, repeated dosing may be required in order to maintain increased receptor activity in a clinical setting.

Variants with reduced cell surface expression appear to fall broadly into two categories, namely those that are not rescuable by any PC, and those that are rescuable by many/all tested PCs. The observation that certain variants appear to be 'unrescuable' implies that the affected residues are directly involved in the binding of small-molecule compounds/PCs; and/or the variants result in major re-orientation/positioning of fundamental determinants of receptor structure, and that these variant receptors are thus 'terminally misfolded'. For example, molecular modelling of the "unrescuable" human GnRHR variants S168R (located in TM4), and S217R (located in TM5) has shown that the exchange of serine (S) with the hydrophilic arginine (R) results in rotation of TM4 and TM5 and a concomitant reorientation of ECL2 which severely impedes the formation of the C14–C200 bridge, preventing ER release presumably due to unsurmountable conformational disruption.[220] In other cases such 'unrescuable' variants could affect specific motifs/regions of the receptor that are critical for trafficking. For example, a frameshift variant, in which the last 83 amino acids of the LHCGR are replaced with 21 different amino acids, ablates a highly conserved $F(X)_6LL$ trafficking motif within helix 8 of the C-terminal tail that is important for ER exit, rendering the variant non-responsive to LHR-Chap rescue.[61]

Further evidence that different Class II LHCGR variants may be handled differently by cellular quality control processes comes from the observation that LHCGR variants A593P (severe loss of cell surface expression) and S616Y (moderate loss of cell surface expression) associate with different ER-resident molecular chaperone proteins.[139] Unlike the WT receptor, coimmunoprecipitation studies demonstrated that the S616Y and A593P variant receptors interact with binding immunoglobulin protein (BiP), a heat shock protein that aids folding and translocation within the ER and in transporting misfolded proteins to the proteasome. Both the WT LHCGR and S616Y variant also interact with protein disulphide isomerase (PDI), an enzyme involved in protein folding in the ER through catalysis of disulphide bonds, while only the A593P variant interacted with the ER stress protein, 94 kDa glucose-regulated protein (Grp94).[139] These observations (in addition to unpublished data from our own studies) suggest that variants may be able to progress to different stages of the folding/maturation pathway, and thus have different degrees/'types' of misfolding.

It would be tempting to hypothesise that more severely misfolded variants may be less responsive to PC rescue, as this may provide a plausible explanation for why some GPCR variants appear to be more receptive to PC rescue than others, despite their locations within the receptor structure being similar. However, it has been observed that intracellularly retained rat LHCGR variants with some degree of cell surface expression (and are thus presumably less severely misfolded) had a greater ability to be rescued by non-selective means (incubation of cells at reduced temperatures to facilitate protein folding) than those that are more profoundly retained (presumably due to more severe misfolding).[221] This is in contrast to the rescue of LHCGR variants with LHR-Chap, where there does not appear to be a correlation between degree of retention and the degree of rescue by PCs.[201] Furthermore, there does not appear to be a correlation between variants with reduced total cellular expression, which may be indicative of a greater degree of misfolding resulting in increased variant receptor degradation (although this is speculative and requires conformation), and their responsiveness to PC rescue.[209] Thus, it is likely that PC

responsiveness is dependent on the specific binding interactions between a PC and receptor variant and how this results in conformational stabilisation rather than the degree of misfolding of the variant receptor. However, further detailed examination of additional variants and cellular quality control processes would be required to elucidate the precise intracellular mechanisms.

The 'rescue efficacy' of most PCs appears to correlate with their binding affinity, which is not surprising as a higher binding affinity would suggest stronger ligand–receptor interactions and thus a greater stabilising effect. As observed previously for other GPCRs/proteins,[222] PC activity appears to require doses higher than the EC_{50} observed in signalling assays for the HPG axis GPCRs. For example the LHCGR agonist and PC, LHR-Chap, exhibited a potency (EC_{50}) of approximately 10 nM for activation of the WT LHCGR (when measuring CRE-luciferase reporter activity), but 'rescue potency' of LHR-Chap at variant LHCGRs was >300 nM.[140] Likewise, CAN 1404 exhibited a potency of 2–6 nM for activation of the WT FSHR (when measured by CRE-luciferase reporter activity or $G\alpha_{16}$-linked inositol phosphate accumulation), but had a rescue potency of 100–300 nM.[209] However, some exceptions to this rule have been noted. For example, when a panel of different small-molecule ligands were tested for FSHR PC activity, LHR-Chap (which does not induce potent activation of the FSHR) exhibited potent FSHR PC activity, while a thiazolidinone compound, CAN 1403, had reasonable potency as an FSHR agonist, but was unable to act as a PC for any of a range of FSHR variants examined (our unpublished observations).

6 | FUNCTIONAL RESCUE OF HORMONE-BINDING/RECEPTOR-SIGNALLING DEFICIENT LHCGR VARIANTS

Thus far, the focus of this review has been on the 'cell surface rescue' of Class II variants of the HPG axis GPCRs. However, functional rescue of gonadotropin receptor variants that have little/no deleterious effect on receptor cell surface localisation but have impairments in hormone-binding (Class III variants) or receptor-signalling (Class IV variants) has also been described. The rationale underlying 'rescue' of these variants hinges on the allosteric agonist property of gonadotropin receptor-targeting small-molecule ligands. The principle behind this rescue is that allosteric agonists can bind to an allosteric site in the GPCR to induce/stabilise an active conformation which overcomes the loss of binding/activation mediated through the orthosteric binding site. With respect to the LHCGR, no stimulation was elicited upon hormone activation of variants C131R and I152T, both of which are located within the LRR region of the ECD and affect hormone binding to the receptor, but the LHCGR allosteric agonist LHR-Chap stimulated a robust response.[201] Likewise, hormone stimulation of the LHCGR variant E354K, which is located in the intramolecular agonist motif of the hinge region critical for transduction of hormone binding in the ECD to activation of the 7-TMD, was severely impaired (reduced potency and a low maximal response). LHR-Chap treatment was able to elicit a greater maximal response at a potency not different from that at the WT receptor.[201]

7 | CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Inactivating variants of GPCRs have been demonstrated to be causative in a range of reproductive disorders, with many variants resulting in reduced receptor expression at the cell surface. Although gene editing could potentially be utilised to 'correct' such variants, these approaches are costly, and specific targeting of variant GPCRs in human patients has not yet been achieved. PCs provide a pharmacological means to restore cell surface expression and function of such retained variants. That several variants of each receptor exhibit increased functionality (hormone responsiveness) following PC rescue[140,147,182,201,209] indicates that many Class II variant receptors retain intrinsic functionality (hormone binding/receptor signalling), which is critical for the therapeutic potential of PCs.

In primary hypogonadism resulting from inactivating LHCGR and FSHR variants, puberty and secondary sexual characteristics can be induced by treatment with sex steroid hormones, yet no effective therapeutic options for promoting fertility currently exist as gametogenesis requires the co-ordinated activities of gonadotropins in addition to sex steroids. The development of PC therapeutics that can restore both sex hormone production and gametogenesis in patients harbouring LHCGR/FSHR variants is promising. However, PC therapy may not be effective at restoring reproductive competence in patients harbouring variants that cause severe loss-of-function resulting in extreme phenotypes (such as complete pseudohermaphroditism). That being said, these compounds still have potential for the therapeutic treatment of females and male patients suffering from milder reproductive dysfunction caused by 'PC responsive' variants. In addition, utilising PCs to restore receptor function (in both primary and secondary hypogonadism) could have the benefit of maintaining the spatial and temporal receptor activation profiles governed by the secretion of endogenous hormones.

An interesting observation to arise from the multiple studies devoted to PC identification is the sheer number of different core scaffolds and functional classes of the small molecules with PC activity. However, this is perhaps not surprising as any cell permeant small-molecule ligand that can interact with a GPCR should stabilise its conformation to some extent and therefore have the potential to act as a PC for destabilising variants of that receptor. Both antagonists and agonists have been utilised as PCs and there are pros and cons to the utilisation of both. Agonists can concurrently activate the variant receptors in addition to restoring their cell surface expression and do not require 'washing out' as with antagonists. However, at the relatively high doses required for PC rescue, they have the potential to induce agonist-mediated desensitisation/downregulation. Thererfore, perhaps a class of pharmacological agent that would make effective PCs would be positive allosteric modulators, which interact with a

receptor and have no effect in isolation, but rather enhance the pharmacodynamic profile of an endogenous hormone ligand. Such an agent could potentially restore variant receptor cell surface expression while simultaneously enhancing and maintaining the spatial and temporal pattern of endogenous hormone responses. Recently there have also been dedicated efforts to synthesise PCs with no intrinsic agonist or antagonist properties.[223,224] These "pure PC" molecules would have discernible advantages in the clinical setting and thus reflect an interesting advancement in the field of PC rescue.

Variants in many of the genes encoding HPG axis receptors follow an autosomal recessive pattern of inheritance. However, in several cases only one variant allele has been detected, suggesting additional unreported variants in other genes or in non-coding portions of the genes that contribute to the patient phenotype. Alternatively, as many GPCRs including the gonadotropin receptors,[143,225] GnRHR,[226] KISS1R[227] and KOR[228,229] are capable of forming homomers and/or heteromers (indeed, in the case of the LHCGR, it has been estimated that only approximately half of plasma membrane localised receptors are in monomeric form[230]), it is possible that intracellularly retained variants may act in a dominant negative manner, resulting in concurrent retention of the WT receptor. Indeed, dominant negative effects of intracellularly retained variants on WT FSHRs, LHCGRs and GnRHRs have been reported.[142,231,232] This could therefore have major implications regarding the frequency of incidence of reproductive dysfunction as a result of variants in these receptors and thus the potential therapeutic scope for PC therapeutics.

In summary, despite multiple studies exploring GPCR-targeting PCs, only a small number of in vivo studies have elaborated upon their potential in the clinical setting. One potential issue is the relative rarity of the identified variants within the population, stifling significant investment in research. However, for many GPCRs multiple small-molecule agonists and antagonists already exist, with many in various stages of clinical development for other indications. Repurposing/repositioning of those molecules that have proven safe and efficacious in human studies could therefore represent a viable repository of potentially effective PCs.

AUTHOR CONTRIBUTIONS

Tarryn Radomsky: Conceptualization; writing – original draft; writing – review and editing. **Ross C. Anderson:** Conceptualization; writing – original draft; writing – review and editing. **Robert P. Millar:** Writing – review and editing. **Claire L. Newton:** Conceptualization; writing – original draft; writing – review and editing.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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REFERENCES

1. Dwyer AA, Smith N, Quinton R. Psychological aspects of congenital hypogonadotropic hypogonadism. *Front Endocrinol*. 2019;10:353.
2. Leach K, Wen A, Cook AE, Sexton PM, Conigrave AD, Christopoulos A. Impact of clinically relevant mutations on the pharmacoregulation and signaling bias of the calcium-sensing receptor by positive and negative allosteric modulators. *Endocrinology*. 2013;154(3):1105-1116.
3. Crowley WF Jr, Filicori M, Spratt DI, Santoro NF. The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. *Recent Prog Horm Res*. 1985;41:473-531.
4. Marques P, Skorupskaite K, Rozario KS, et al. Physiology of GnRH and gonadotropin secretion. In: Feingold KR, Anawalt B, Blackman MR, et al., eds. *Endotext [Internet]*. MDText.com, Inc; 2000. doi:10.1016/0306-4522(92)90423-y
5. Seminara SB, Messager S, Chatzidaki EE, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med*. 2003;349(17):1614-1627.
6. Navarro VM, Fernández-Fernández R, Castellano JM, et al. Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. *J Physiol*. 2004;561(Pt 2):379-386.
7. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA*. 2003;100(19):10972-10976.
8. Navarro VM. Metabolic regulation of kisspeptin the link between energy balance and reproduction. *Nat Rev Endocrinol*. 2020;16(8):407-420.
9. Goodman RL, Lehman MN, Smith JT, et al. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin a and neurokinin B. *Endocrinology*. 2007;148(12):5752-5760.
10. Topaloglu AK, Reimann F, Guclu M, et al. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for neurokinin B in the central control of reproduction. *Nat Genet*. 2009;41(3):354-358.
11. Gianetti E, Tusset C, Noel SD, et al. TAC3/TACR3 mutations reveal preferential activation of gonadotropin-releasing hormone release by neurokinin B in neonatal life followed by reversal in adulthood. *J Clin Endocrinol Metab*. 2010;95(6):2857-2867.
12. Young J, Bouligand J, Francou B, et al. TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. *J Clin Endocrinol Metab*. 2010;95(5):2287-2295.
13. Young J, George JT, Tello JA, et al. Kisspeptin restores pulsatile LH secretion in patients with neurokinin B signaling deficiencies: physiological, pathophysiological and therapeutic implications. *Neuroendocrinology*. 2013;97(2):193-202.
14. Francou B, Bouligand J, Voican A, et al. Normosmic congenital hypogonadotropic hypogonadism due to TAC3/TACR3 mutations: characterization of neuroendocrine phenotypes and novel mutations. *PLoS One*. 2011;6(10):e25614.
15. Ramaswamy S, Seminara SB, Ali B, Ciofi P, Amin NA, Plant TM. Neurokinin B stimulates GnRH release in the male monkey (*Macaca mulatta*) and is colocalized with kisspeptin in the arcuate nucleus. *Endocrinology*. 2010;151(9):4494-4503.
16. Navarro VM, Gottsch ML, Wu M, et al. Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. *Endocrinology*. 2011;152(11):4265-4275.
17. Clarke IJ, Li Q, Henry BA, Millar RP. Continuous Kisspeptin restores luteinizing hormone pulsatility following cessation by a neurokinin B antagonist in female sheep. *Endocrinology*. 2018;159(2):639-646.

18. Navarro VM, Castellano JM, McConkey SM, et al. Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. *Am J Physiol Endocrinol Metab*. 2011;300(1):E202-E210.
19. Anderson RA, Cormier J, Thieroff-Ekerdt R, et al. Pharmacodynamic activity of the novel neurokinin-3 receptor antagonist SJX-653 in healthy men. *J Clin Endocrinol Metab*. 2020;105(12):e4857-e4865.
20. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Neurokinin 3 receptor antagonism reveals roles for neurokinin B in the regulation of gonadotropin secretion and hot flashes in postmenopausal women. *Neuroendocrinology*. 2018;106(2):148-157.
21. Prague JK, Abbara A, Comninos AN, et al. Neurokinin 3 receptor antagonists do not increase FSH or estradiol secretion in menopausal women. *J Endocr Soc*. 2020;4(2):p. bvz009.
22. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Neurokinin 3 receptor antagonism decreases gonadotropin and testosterone secretion in healthy men. *Clin Endocrinol*. 2017;87(6):748-756.
23. Vuong C, van Uum SHM, O'Dell LE, Lutfy K, Friedman TC. The effects of opioids and opioid analogs on animal and human endocrine systems. *Endocr Rev*. 2010;31(1):98-132.
24. Li S, Pelletier G. Opioid regulation of gonadotropin-releasing hormone gene expression in the male rat brain as studied by in situ hybridization. *Neuroreport*. 1993;4(3):331-333.
25. Uenoyama Y, Tsuchida H, Nagae M, Inoue N, Tsukamura H. Opioidergic pathways and kisspeptin in the regulation of female reproduction in mammals. *Front Neurosci*. 2022;16:958377.
26. Nakahara T, Uenoyama Y, Iwase A, et al. Chronic peripheral administration of kappa-opioid receptor antagonist advances puberty onset associated with acceleration of pulsatile luteinizing hormone secretion in female rats. *J Reprod Dev*. 2013;59(5):479-484.
27. Genazzani AD, Gastaldi M, Petraglia F, et al. Naltrexone administration modulates the neuroendocrine control of luteinizing hormone secretion in hypothalamic amenorrhoea. *Hum Reprod*. 1995;10(11):2868-2871.
28. Fruzzetti F, Bersi C, Parrini D, Ricci C, Genazzani AR. Effect of long-term naltrexone treatment on endocrine profile, clinical features, and insulin sensitivity in obese women with polycystic ovary syndrome. *Fertil Steril*. 2002;77(5):936-944.
29. Simpson ER, Mahendroo MS, Means GD, et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr Rev*. 1994;15(3):342-355.
30. Cheng CY, Mruk DD. A local autocrine axis in the testes that regulates spermatogenesis. *Nat Rev Endocrinol*. 2010;6(7):380-395.
31. Young J, Xu C, Papadakis GE, et al. Clinical management of congenital hypogonadotropic hypogonadism. *Endocr Rev*. 2019;40(2):669-710.
32. Topaloglu AK, Tello JA, Kotan LD, et al. Inactivating KISS1 mutation and hypogonadotropic hypogonadism. *N Engl J Med*. 2012;366:629-635.
33. Beranova M, Oliveira LM, Bédécarrats GY, et al. Prevalence, phenotypic spectrum, and modes of inheritance of gonadotropin-releasing hormone receptor mutations in idiopathic hypogonadotropic hypogonadism. *J Clin Endocrinol Metab*. 2001;86(4):1580-1588.
34. Chan YM, de Guillebon A, Lang-Muritano M, et al. GNRH1 mutations in patients with idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA*. 2009;106(28):11703-11708.
35. Bruysters M, Christin-Maitre S, Verhoef-Post M, et al. A new LH receptor splice mutation responsible for male hypogonadism with subnormal sperm production in the propositus, and infertility with regular cycles in an affected sister. *Hum Reprod*. 2008;23(8):1917-1923.
36. el-Awady MK, Temtamy SA, Salam MA, Gad YZ. Familial Leydig cell hypoplasia as a cause of male pseudohermaphroditism. *Hum Hered*. 1987;37(1):36-40.
37. Gromoll J, Eiholzer U, Nieschlag E, Simoni M. Male hypogonadism caused by homozygous deletion of exon 10 of the luteinizing hormone (LH) receptor: differential action of human chorionic gonadotropin and LH. *J Clin Endocrinol Metab*. 2000;85(6):2281-2286.
38. Gromoll J, Schulz A, Borta H, et al. Homozygous mutation within the conserved Ala-Phe-Asn-Glu-Thr motif of exon 7 of the LH receptor causes male pseudohermaphroditism. *Eur J Endocrinol*. 2002;147:597-608.
39. Kossack N, Troppmann B, Richter-Unruh A, Kleinau G, Gromoll J. Aberrant transcription of the LHCGR gene caused by a mutation in exon 6A leads to Leydig cell hypoplasia type II. *Mol Cell Endocrinol*. 2013;366(1):59-67.
40. Kremer H, Kraaij R, Toledo SPA, et al. Male pseudohermaphroditism due to a homozygous missense mutation of the luteinizing hormone receptor gene. *Nat Genet*. 1995;9(2):160-164.
41. Latronico AC, Anasti J, Arnhold IJP, et al. Testicular and ovarian resistance to luteinizing hormone caused by inactivating mutations of the luteinizing hormone–receptor gene. *New Engl J Med*. 1996;334(8):507-512.
42. Latronico AC, Chai Y, Arnhold IJP, Liu X, Mendonca BB, Segaloff DL. A homozygous microdeletion in helix 7 of the luteinizing hormone receptor associated with familial testicular and ovarian resistance is due to both decreased cell surface expression and impaired effector activation by the cell surface receptor. *Mol Endocrinol*. 1998;12(3):442-450.
43. Laue L, Wu SM, Kudo M, et al. A nonsense mutation of the human luteinizing hormone receptor gene in Leydig cell hypoplasia. *Hum Mol Genet*. 1995;4(8):1429-1433.
44. Laue LL, Wu SM, Kudo M, et al. Compound heterozygous mutations of the luteinizing hormone receptor gene in Leydig cell hypoplasia. *Mol Endocrinol*. 1996;10(8):987-997.
45. Leung MY-K, Steinbach PJ, Bear D, et al. Biological effect of a novel mutation in the third leucine-rich repeat of human luteinizing hormone receptor. *Mol Endocrinol*. 2006;20(10):2493-2503.
46. Leung MYK, al-Muslim O, Wu SM, et al. A novel missense homozygous inactivating mutation in the fourth transmembrane helix of the luteinizing hormone receptor in Leydig cell hypoplasia. *Am J Med Genet A*. 2004;130A(2):146-153.
47. Martens JWM, Lumbroso S, Verhoef-Post M. Mutant luteinizing hormone receptors in a compound heterozygous patient with complete Leydig cell hypoplasia: abnormal processing causes signaling deficiency. *J Clin Endocrinol Metab*. 2002;87(6):2506-2513.
48. Martens JWM, Verhoef-Post M, Abelin N, et al. A homozygous mutation in the luteinizing hormone receptor causes partial Leydig cell hypoplasia: correlation between receptor activity and phenotype. *Mol Endocrinol*. 1998;12(6):775-784.
49. Misrahi M, Meduri G, Pissard S, et al. Comparison of Immunocytochemical and molecular features with the phenotype in a case of incomplete male pseudohermaphroditism associated with a mutation of the luteinizing hormone receptor. *J Clin Endocrinol Metab*. 1997;82(7):2159-2165.
50. Pals-Rylaarsdam R, Liu G, Brickman W, et al. A novel double mutation in the luteinizing hormone receptor in a kindred with familial Leydig cell hypoplasia and male pseudohermaphroditism. *Endocr Res*. 2005;31(4):307-323.
51. Qiao J, Han B, Liu BL, et al. A splice site mutation combined with a novel missense mutation of *LHCGR* cause male pseudohermaphroditism. *Hum Mutat*. 2009;30(9):E855-E865.
52. Richter-Unruh A, Korsch E, Hiort O, Holterhus PM, Themmen AP, Wudy SA. Novel insertion frameshift mutation of the LH receptor gene: problematic clinical distinction of Leydig cell hypoplasia from enzyme defects primarily affecting testosterone biosynthesis. *Eur J Endocrinol*. 2005;152(2):255-259.
53. Richter-Unruh A, Verhoef-Post M, Malak S, Homoki J, Hauffa BP, Themmen APN. Leydig cell hypoplasia: absent luteinizing hormone

receptor cell surface expression caused by a novel homozygous mutation in the extracellular domain. *J Clin Endocrinol Metab*. 2004; 89(10):5161-5167.
54. Richter-Unruh A, Martens JWM, Verhoef-Post M, et al. Leydig cell hypoplasia: cases with new mutations, new polymorphisms and cases without mutations in the luteinizing hormone receptor gene. *Clin Endocrinol*. 2002;56(1):103-112.
55. Salameh W, Choucair M, Guo TB, et al. Leydig cell hypoplasia due to inactivation of luteinizing hormone receptor by a novel homozygous nonsense truncation mutation in the seventh transmembrane domain. *Mol Cell Endocrinol*. 2005;229(1–2):57-64.
56. Stavrou SS, Zhu YS, Cai LQ, et al. A novel mutation of the human luteinizing hormone receptor in 46XY and 46XX sisters. *J Clin Endocrinol Metab*. 1998;83(6):2091-2098.
57. Toledo SP, Brunner HG, Kraaij R, et al. An inactivating mutation of the luteinizing hormone receptor causes amenorrhea in a 46,XX female. *J Clin Endocrinol Metab*. 1996;81(11):3850-3854.
58. Wu S-M, Hallermeier KM, Laue L, et al. Inactivation of the luteinizing hormone/chorionic gonadotropin receptor by an insertional mutation in Leydig cell hypoplasia. *Mol Endocrinol*. 1998;12(11):1651-1660.
59. Yariz KO, Walsh T, Uzak A, et al. Inherited mutation of the luteinizing hormone/choriogonadotropin receptor (LHCGR) in empty follicle syndrome. *Fertil Steril*. 2011;96(2):e125-e130.
60. Kossack N, Simoni M, Richter-Unruh A, Themmen APN, Gromoll J. Mutations in a novel, cryptic exon of the luteinizing hormone/chorionic gonadotropin receptor gene cause male pseudohermaphroditism. *PLoS Med*. 2008;5(4):e88.
61. Rivero-Muller A, Potorac I, Pintiaux A, et al. A novel inactivating mutation of the LH/chorionic gonadotrophin receptor with impaired membrane trafficking leading to Leydig cell hypoplasia type 1. *Eur J Endocrinol*. 2015;172(6):K27-K36.
62. Kotlar TJ, Young RH, Albanese C, Crowley WF Jr, Scully RE, Jameson JL. A mutation in the follicle-stimulating hormone receptor occurs frequently in human ovarian sex cord tumors. *J Clin Endocrinol Metab*. 1997;82(4):1020-1026.
63. Bramble MS, Goldstein EH, Lipson A, et al. A novel follicle-stimulating hormone receptor mutation causing primary ovarian failure: a fertility application of whole exome sequencing. *Hum Reprod*. 2016;31(4):905-914.
64. Hugon-Rodin J, Sonigo C, Gompel A, et al. First mutation in the FSHR cytoplasmic tail identified in a non-pregnant woman with spontaneous ovarian hyperstimulation syndrome. *BMC Med Genet*. 2017;18(1):44.
65. Allen LA, Achermann JC, Pakarinen P, et al. A novel loss of function mutation in exon 10 of the FSH receptor gene causing hypergonadotrophic hypogonadism: clinical and molecular characteristics. *Hum Reprod*. 2003;18(2):251-256.
66. Aittomaki K, Lucena JL, Pakarinen P, et al. Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell*. 1995;82(6):959-968.
67. Beau I, Touraine P, Meduri G, et al. A novel phenotype related to partial loss of function mutations of the follicle stimulating hormone receptor. *J Clin Invest*. 1998;102(7):1352-1359.
68. Nakamura Y, Maekawa R, Yamagata Y, Tamura I, Sugino N. A novel mutation in exon8 of the follicle-stimulating hormone receptor in a woman with primary amenorrhea. *Gynecol Endocrinol*. 2008;24(12): 708-712.
69. Liu H, Xu X, Han T, et al. A novel homozygous mutation in the FSHR gene is causative for primary ovarian insufficiency. *Fertil Steril*. 2017; 108(6):1050-1055.e2.
70. Touraine P, Beau I, Gougeon A, et al. New natural inactivating mutations of the follicle-stimulating hormone receptor: correlations between receptor function and phenotype. *Mol Endocrinol*. 1999; 13(11):1844-1854.
71. Doherty E, Pakarinen P, Tiitinen A, et al. A novel mutation in the FSH receptor inhibiting signal transduction and causing primary ovarian failure. *J Clin Endocrinol Metab*. 2002;87(3):1151-1155.
72. Katari S, Wood-Trageser MA, Jiang H, et al. Novel inactivating mutation of the FSH receptor in two siblings of Indian origin with premature ovarian failure. *J Clin Endocrinol Metab*. 2015;100(6): 2154-2157.
73. Kuechler A, Hauffa BP, Köninger A, et al. An unbalanced translocation unmasks a recessive mutation in the follicle-stimulating hormone receptor (FSHR) gene and causes FSH resistance. *Eur J Hum Genet*. 2010;18(6):656-661.
74. Sassi A, Désir J, Janssens V, et al. Novel inactivating follicle-stimulating hormone receptor mutations in a patient with premature ovarian insufficiency identified by next-generation sequencing gene panel analysis. *F S Rep*. 2020;1(3):193-201.
75. He WB, du J, Yang XW, et al. Novel inactivating mutations in the FSH receptor cause premature ovarian insufficiency with resistant ovary syndrome. *Reprod Biomed Online*. 2019;38(3):397-406.
76. Yoo S, Yoon JY, Keum C, Cheon CK. The first case of novel variants of the FSHR mutation causing primary amenorrhea in 2 siblings in Korea. *Ann Pediatr Endocrinol Metab*. 2023;28(1):54-60.
77. Chen X, Chen L, Wang Y, et al. Identification and characterization of novel compound heterozygous variants in FSHR causing primary ovarian insufficiency with resistant ovary syndrome. *Front Endocrinol*. 2022;13:1013894.
78. Achrekar SK, Modi DN, Meherji PK, Patel ZM, Mahale SD. Follicle stimulating hormone receptor gene variants in women with primary and secondary amenorrhea. *J Assist Reprod Genet*. 2010;27(6): 317-326.
79. Banerjee AA, Achrekar SK, Joseph S, Pathak BR, Mahale SD. Functional characterization of two naturally occurring mutations V 221 G and T 449 N in the follicle stimulating hormone receptor. *Mol Cell Endocrinol*. 2017;440:69-79.
80. Desai SS, Achrekar SK, Sahasrabuddhe KA, et al. Functional characterization of two naturally occurring mutations ($Val^{514}Ala$ and $Ala^{575}Val$) in follicle-stimulating hormone receptor. *J Clin Endocrinol Metab*. 2015;100(4):E638-E645.
81. França MM, Lerario AM, Funari MFA, et al. A novel homozygous missense FSHR variant associated with hypergonadotropic hypogonadism in two siblings from a Brazilian family. *Sex Dev*. 2017;11(3): 137-142.
82. Gromoll J, Simoni M, Nordhoff V, Behre HM, de Geyter C, Nieschlag E. Functional and clinical consequences of mutations in the FSH receptor. *Mol Cell Endocrinol*. 1996;125(1-2):177-182.
83. Gudermann T, Nurwakagari P, Ben-Menahem D. Hormone binding to the follicle-stimulating hormone receptor—crystal clear! *Exp Clin Endocrinol Diabetes*. 2005;113(5):245-247.
84. Khor S, Lyu Q, Kuang Y, Lu X. Novel FSHR variants causing female resistant ovary syndrome. *Mol Genet Genomic Med*. 2020;8(2): e1082.
85. Meduri G, Touraine P, Beau I, et al. Delayed puberty and primary amenorrhea associated with a novel mutation of the human follicle-stimulating hormone receptor: clinical, histological, and molecular studies. *J Clin Endocrinol Metabm*. 2003;88(8):3491-3498.
86. Orio F Jr, Ferrarini E, Cascella T, et al. Genetic analysis of the follicle stimulating hormone receptor gene in women with polycystic ovary syndrome. *J Endocrinol Invest*. 2006;29(11):975-982.
87. Uchida S, Uchida H, Maruyama T, et al. Molecular analysis of a mutated FSH receptor detected in a patient with spontaneous ovarian hyperstimulation syndrome. *PLoS One*. 2013;8(9):e75478.
88. Zheng J, Mao J, Cui M, et al. Novel FSHbeta mutation in a male patient with isolated FSH deficiency and infertility. *Eur J Med Genet*. 2017;60(6):335-339.
89. Huhtaniemi IT, Themmen AP. Mutations in human gonadotropin and gonadotropin-receptor genes. *Endocrine*. 2005;26(3):207-217.

90. Themmen APN, Huhtaniemi IT. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary–gonadal function. *Endocr Rev*. 2000;21(5):551-583.
91. Desai SS, Roy BS, Mahale SD. Mutations and polymorphisms in FSH receptor: functional implications in human reproduction. *Reproduction*. 2013;146(6):R235-R248.
92. Tapanainen JS, Aittomäki K, Min J, Vaskivuo T, Huhtaniemi IT. Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. *Nat Genet*. 1997;15(2):205-206.
93. Lofrano-Porto A, Barra GB, Giacomini LA, et al. Luteinizing hormone beta mutation and hypogonadism in men and women. *N Engl J Med*. 2007;357(9):897-904.
94. Layman LC, Lee EJ, Peak DB, et al. Delayed puberty and hypogonadism caused by mutations in the follicle-stimulating hormone beta-subunit gene. *N Engl J Med*. 1997;337(9):607-611.
95. Layman LC, Porto AL, Xie J, et al. FSH beta gene mutations in a female with partial breast development and a male sibling with normal puberty and azoospermia. *J Clin Endocrinol Metab*. 2002;87(8):3702-3707.
96. Kottler ML, Chou YY, Chabre O, et al. A new FSHbeta mutation in a 29-year-old woman with primary amenorrhea and isolated FSH deficiency: functional characterization and ovarian response to human recombinant FSH. *Eur J Endocrinol*. 2010;162(3):633-641.
97. Matthews CH, Borgato S, Beck-Peccoz P, et al. Primary amenorrhoea and infertility due to a mutation in the beta-subunit of follicle-stimulating hormone. *Nat Genet*. 1993;5(1):83-86.
98. Berger K, Souza H, Brito VN, d'Alva CB, Mendonca BB, Latronico AC. Clinical and hormonal features of selective follicle-stimulating hormone (FSH) deficiency due to FSH beta-subunit gene mutations in both sexes. *Fertil Steril*. 2005;83(2):466-470.
99. Lindstedt G, Nyström E, Matthews C, Ernest I, Janson PO, Chatterjee K. Follitropin (FSH) deficiency in an infertile male due to FSHbeta gene mutation. A syndrome of normal puberty and virilization but underdeveloped testicles with azoospermia, low FSH but high lutropin and normal serum testosterone concentrations. *Clin Chem Lab Med*. 1998;36(8):663-665.
100. Phillip M, Arbelle JE, Segev Y, Parvari R. Male hypogonadism due to a mutation in the gene for the beta-subunit of follicle-stimulating hormone. *N Engl J Med*. 1998;338(24):1729-1732.
101. Weiss J, Axelrod L, Whitcomb RW, Harris PE, Crowley WF, Jameson JL. Hypogonadism caused by a single amino acid substitution in the beta subunit of luteinizing hormone. *N Engl J Med*. 1992;326(3):179-183.
102. Valdes-Socin H, Salvi R, Daly AF, et al. Hypogonadism in a patient with a mutation in the luteinizing hormone beta-subunit gene. *N Engl J Med*. 2004;351(25):2619-2625.
103. Song JW, Hwang HJ, Lee CM, et al. Hypogonadotrophic hypogonadism due to a mutation in the luteinizing hormone beta-subunit gene. *Korean J Intern Med*. 2018;33(3):638-641.
104. Achard C, Courtillot C, Lahuna O, et al. Normal spermatogenesis in a man with mutant luteinizing hormone. *N Engl J Med*. 2009;361(19):1856-1863.
105. Basciani S, Watanabe M, Mariani S, et al. Hypogonadism in a patient with two novel mutations of the luteinizing hormone beta-subunit gene expressed in a compound heterozygous form. *J Clin Endocrinol Metab*. 2012;97(9):3031-3038.
106. Richard-Eaglin A. Male and female hypogonadism. *Nurs Clin North Am*. 2018;53(3):395-405.
107. Sriram K, Insel PA. G protein-coupled receptors as targets for approved drugs: how many targets and how many drugs? *Mol Pharmacol*. 2018;93(4):251-258.
108. Hauser AS, Attwood MM, Rask-Andersen M, Schiöth HB, Gloriam DE. Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov*. 2017;16(12):829-842.
109. Stojilkovic SS, Reinhart J, Catt KJ. Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. *Endocr Rev*. 1994;15:462-499.
110. Fredriksson R, Lagerström MC, Schiöth HB. Expansion of the superfamily of G-protein-coupled receptors in chordates. *Ann N Y Acad Sci*. 2005;1040:89-94.
111. Jiang X, Liu H, Chen X, et al. Structure of follicle-stimulating hormone in complex with the entire ectodomain of its receptor. *Proc Natl Acad Sci USA*. 2012;109(31):12491-12496.
112. Bruser A, Schulz A, Rothemund S, et al. The activation mechanism of glycoprotein hormone receptors with implications in the cause and therapy of endocrine diseases. *J Biol Chem*. 2016;291(2):508-520.
113. Schulze A, Kleinau G, Neumann S, Scheerer P, Schöneberg T, Brüser A. The intramolecular agonist is obligate for activation of glycoprotein hormone receptors. *FASEB J*. 2020;34(8):11243-11256.
114. Tao YX. Inactivating mutations of G protein-coupled receptors and diseases: structure-function insights and therapeutic implications. *Pharmacol Ther*. 2006;111(3):949-973.
115. Sahni N, Yi S, Taipale M, et al. Widespread macromolecular interaction perturbations in human genetic disorders. *Cell*. 2015;161(3):647-660.
116. Ulloa-Aguirre A, Zariñán T, Gutiérrez-Sagal R, Tao YX. Targeting trafficking as a therapeutic avenue for misfolded GPCRs leading to endocrine diseases. *Front Endocrinol*. 2022;13.
117. Newton CL, Anderson RC, Katz AA, Millar RP. Loss-of-function mutations in the human luteinizing hormone receptor predominantly cause intracellular retention. *Endocrinology*. 2016;157(11):4364-4377.
118. Oksche A, Rosenthal W. The molecular basis of nephrogenic diabetes insipidus. *J Mol Med*. 1998;76(5):326-337.
119. Ulloa-Aguirre A, Zariñán T, Dias JA, Conn PM. Mutations in G protein-coupled receptors that impact receptor trafficking and reproductive function. *Mol Cell Endocrinol*. 2014;382(1):411-423.
120. Braakman I, Hebert DN. Protein folding in the endoplasmic reticulum. *Cold Spring Harb Perspect Biol*. 2013;5(5):a013201.
121. Hetz C, Zhang K, Kaufman RJ. Mechanisms, regulation and functions of the unfolded protein response. *Nat Rev Mol Cell Biol*. 2020;21(8):421-438.
122. Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol*. 2008;9(12):944-957.
123. Piersma D, Berns EMJJ, Verhoef-Post M, et al. A common polymorphism renders the luteinizing hormone receptor protein more active by improving signal peptide function and predicts adverse outcome in breast cancer patients. *J Clin Endocrinol Metab*. 2006;91(4):1470-1476.
124. Finch AR, Sedgley KR, Armstrong SP, Caunt CJ, McArdle C. Trafficking and signalling of gonadotrophin-releasing hormone receptors: an automated imaging approach. *Br J Pharmacol*. 2010;159(4):751-760.
125. Conn PM, Janovick JA. Pharmacoperone identification for therapeutic rescue of misfolded mutant proteins. *Front Endocrinol*. 2011;2(6).
126. Conn PM, Janovick JA, Brothers SP, Knollman PE. 'Effective inefficiency': cellular control of protein trafficking as a mechanism of post-translational regulation. *J Endocrinol*. 2006;190(1):13-16.
127. Ulloa-Aguirre A, Zariñán T, Jardón-Valadez E. Misfolded G protein-coupled receptors and endocrine disease. Molecular mechanisms and therapeutic prospects. *Int J Mol Sci*. 2021;22(22).
128. Flanagan CA, Manilall A. Gonadotropin-releasing hormone (GnRH) receptor structure and GnRH binding. *Front Endocrinol*. 2017;8:274.
129. Janovick JA, Knollman PE, Brothers SP, Ayala-Yáñez R, Aziz AS, Conn PM. Regulation of G protein-coupled receptor trafficking by inefficient plasma membrane expression: molecular basis of an evolved strategy. *J Biol Chem*. 2006;281(13):8417-8425.

130. Jardón-Valadez E, Aguilar-Rojas A, Maya-Núñez G, et al. Conformational effects of Lys191 in the human GnRH receptor: mutagenesis and molecular dynamics simulations studies. *J Endocrinol*. 2009; 201(2):297-307.
131. Soderlund D, Canto P, de la Chesnaye E, Ulloa-Aguirre A, Méndez JP. A novel homozygous mutation in the second transmembrane domain of the gonadotrophin releasing hormone receptor gene. *Clin Endocrinol*. 2001;54(4):493-498.
132. Janovick JA, Conn PM. Salt bridge integrates GPCR activation with protein trafficking. *Proc Natl Acad Sci USA*. 2010;107(9):4454-4458.
133. Janovick JA, Goulet M, Bush E, Greer J, Wettlaufer DG, Conn PM. Structure-activity relations of successful pharmacologic chaperones for rescue of naturally occurring and manufactured mutants of the gonadotropin-releasing hormone receptor. *J Pharmacol Exp Ther*. 2003;305(2):608-614.
134. Janovick JA, Maya-Nunez G, Conn PM. Rescue of hypogonadotropic hypogonadism-causing and manufactured GnRH receptor mutants by a specific protein-folding template: misrouted proteins as a novel disease etiology and therapeutic target. *J Clin Endocrinol Metab*. 2002;87(7):3255-3262.
135. Knollman PE, Janovick JA, Brothers SP, Conn PM. Parallel regulation of membrane trafficking and dominant-negative effects by misrouted gonadotropin-releasing hormone receptor mutants. *J Biol Chem*. 2005;280(26):24506-24514.
136. Manilall A, Stander BA, Madziva MT, Millar RP, Flanagan CA. $Glu^{(2.53(90))}$ of the GnRH receptor is part of the conserved G protein-coupled receptor structure and does not form a salt-bridge with $Lys^{(3.32(121))}$. *Mol Cell Endocrinol*. 2019;481:53-61.
137. Houck SA, Ren HY, Madden VJ, et al. Quality control autophagy degrades soluble ERAD-resistant conformers of the misfolded membrane protein GnRHR. *Mol Cell*. 2014;54(1):166-179.
138. Segaloff DL, Ascoli M. The lutropin/choriogonadotropin receptor … 4 years later. *Endocr Rev*. 1993;14(3):324-347.
139. Mizrachi D, Segaloff DL. Intracellularly located misfolded glycoprotein hormone receptors associate with different chaperone proteins than their cognate wild-type receptors. *Mol Endocrinol*. 2004;18(7): 1768-1777.
140. Newton CL, Whay AM, McArdle CA, et al. Rescue of expression and signaling of human luteinizing hormone G protein-coupled receptor mutants with an allosterically binding small-molecule agonist. *Proc Natl Acad Sci*. 2011;108(17):7172-7176.
141. Agwuegbo UC, Jonas KC. Molecular and functional insights into gonadotropin hormone receptor dimerization and oligomerization. *Minerva Ginecol*. 2018;70(5):539-548.
142. Tao YX, Johnson NB, Segaloff DL. Constitutive and agonist-dependent self-association of the cell surface human lutropin receptor. *J Biol Chem*. 2004;279(7):5904-5914.
143. Guan R, Feng X, Wu X, et al. Bioluminescence resonance energy transfer studies reveal constitutive dimerization of the human lutropin receptor and a lack of correlation between receptor activation and the propensity for dimerization. *J Biol Chem*. 2009;284(12): 7483-7494.
144. Guran T, Tolhurst G, Bereket A, et al. Hypogonadotropic hypogonadism due to a novel missense mutation in the first extracellular loop of the neurokinin B receptor. *J Clin Endocrinol Metab*. 2009; 94(10):3633-3639.
145. Tusset C, Noel SD, Trarbach EB, et al. Mutational analysis of TAC3 and TACR3 genes in patients with idiopathic central pubertal disorders. *Arq Bras Endocrinol Metabol*. 2012;56(9):646-652.
146. Aoyama K, Mizuno H, Tanaka T, et al. Molecular genetic and clinical delineation of 22 patients with congenital hypogonadotropic hypogonadism. *J Pediatr Endocrinol Metab*. 2017;30(10):1111-1118.
147. Anderson RC, Hanyroup S, Song YB, et al. Functional rescue of inactivating mutations of the human neurokinin 3 receptor using pharmacological chaperones. *Int J Mol Sci*. 2022;23(9).
148. Wacker JL, Feller DB, Tang XB, et al. Disease-causing mutation in GPR54 reveals the importance of the second intracellular loop for class A G-protein-coupled receptor function. *J Biol Chem*. 2008; 283(45):31068-31078.
149. Shaw ND, Seminara SB, Welt CK, et al. Expanding the phenotype and genotype of female GnRH deficiency. *J Clin Endocrinol Metab*. 2011;96(3):E566-E576.
150. Teles MG, Trarbach EB, Noel SD, et al. A novel homozygous splice acceptor site mutation of KISS1R in two siblings with normosmic isolated hypogonadotropic hypogonadism. *Eur J Endocrinol*. 2010; 163(1):29-34.
151. Nimri R, Lebenthal Y, Lazar L, et al. A novel loss-of-function mutation in *GPR54/KISS1R* leads to hypogonadotropic hypogonadism in a highly consanguineous family. *J Clin Endocrinol Metabol*. 2011; 96(3):E536-E545.
152. Sykiotis GP, Plummer L, Hughes VA, et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. *Proc Natl Acad Sci USA*. 2010;107(34):15140-15144.
153. Miraoui H, Dwyer AA, Sykiotis GP, et al. Mutations in FGF17, IL17RD, DUSP6, SPRY4, and FLRT3 are identified in individuals with congenital hypogonadotropic hypogonadism. *Am J Hum Genet*. 2013;92(5):725-743.
154. Brioude F, Bouligand J, Francou B, et al. Two families with normosmic congenital hypogonadotropic hypogonadism and biallelic mutations in KISS1R (KISS1 receptor): clinical evaluation and molecular characterization of a novel mutation. *PLoS One*. 2013;8(1):e53896.
155. Semple RK, Achermann JC, Ellery J, et al. Two novel missense mutations in g protein-coupled receptor 54 in a patient with hypogonadotropic hypogonadism. *J Clin Endocrinol Metab*. 2005;90(3): 1849-1855.
156. Shahab M, Lippincott M, Chan YM, et al. Discordance in the dependence on Kisspeptin signaling in mini puberty vs adolescent puberty: human genetic evidence. *J Clin Endocrinol Metab*. 2018;103(4):1273-1276.
157. Lanfranco F, Gromoll J, von Eckardstein S, Herding EM, Nieschlag E, Simoni M. Role of sequence variations of the GnRH receptor and G protein-coupled receptor 54 gene in male idiopathic hypogonadotropic hypogonadism. *Eur J Endocrinol*. 2005;153(6):845-852.
158. Breuer O, Abdulhadi-Atwan M, Zeligson S, et al. A novel severe N-terminal splice site KISS1R gene mutation causes hypogonadotropic hypogonadism but enables a normal development of neonatal external genitalia. *Eur J Endocrinol*. 2012;167(2):209-216.
159. Demirbilek H, Ozbek MN, Demir K, et al. Normosmic idiopathic hypogonadotropic hypogonadism due to a novel homozygous nonsense c.C969A (p.Y323X) mutation in the KISS1R gene in three unrelated families. *Clin Endocrinol*. 2015;82(3):429-438.
160. Francou B, Paul C, Amazit L, et al. Prevalence of KISS1 receptor mutations in a series of 603 patients with normosmic congenital hypogonadotrophic hypogonadism and characterization of novel mutations: a single-centre study. *Hum Reprod*. 2016;31(6):1363-1374.
161. Geng D, Zhang H, Liu X, et al. Identification of KISS1R gene mutations in disorders of non-obstructive azoospermia in the northeast population of China. *J Clin Lab Anal*. 2020;34(4):e23139.
162. Moalla M, Hadj Kacem F, al-Mutery AF, et al. Nonstop mutation in the Kisspeptin 1 receptor (KISS1R) gene causes normosmic congenital hypogonadotropic hypogonadism. *J Assist Reprod Genet*. 2019; 36(6):1273-1280.
163. Tenenbaum-Rakover Y, Commenges-Ducos M, Iovane Á, Aumas C, Admoni O, de Roux N. Neuroendocrine phenotype analysis in five patients with isolated hypogonadotropic hypogonadism due to a L102P inactivating mutation of GPR54. *J Clin Endocrinol Metab*. 2007;92(3):1137-1144.
164. Nimri R, Lebenthal Y, Lazar L, et al. A novel loss-of-function mutation in GPR54/KISS1R leads to hypogonadotropic hypogonadism in

a highly consanguineous family. *J Clin Endocrinol Metab*. 2011;96(3):E536-E545.
165. Butelman ER, Yuferov V, Kreek MJ. κ-opioid receptor/dynorphin system: genetic and pharmacotherapeutic implications for addiction. *Trends Neurosci*. 2012;35(10):587-596.
166. Xu K, Seo D, Hodgkinson C, Hu Y, Goldman D, Sinha R. A variant on the kappa opioid receptor gene (OPRK1) is associated with stress response and related drug craving, limbic brain activation and cocaine relapse risk. *Transl Psychiatry*. 2013;3(8):e292.
167. Xu W, Campillo M, Pardo L, Kim de Riel J, Liu-Chen LY. The seventh transmembrane domains of the delta and kappa opioid receptors have different accessibility patterns and interhelical interactions. *Biochemistry*. 2005;44(49):16014-16025.
168. Papp E, Csermely P. Chemical chaperones: mechanisms of action and potential use. *Handb Exp Pharmacol*. 2006;172:405-416.
169. Bernier V, Lagacé M, Bichet DG, Bouvier M. Pharmacological chaperones: potential treatment for conformational diseases. *Trends Endocrinol Metab*. 2004;15(5):222-228.
170. Tao YX, Conn PM. Chaperoning G protein-coupled receptors: from cell biology to therapeutics. *Endocr Rev*. 2014;35(4):602-647.
171. Mijnders M, Kleizen B, Braakman I. Correcting CFTR folding defects by small-molecule correctors to cure cystic fibrosis. *Curr Opin Pharmacol*. 2017;34:83-90.
172. Carlile GW, Robert R, Goepp J, et al. Ibuprofen rescues mutant cystic fibrosis transmembrane conductance regulator trafficking. *J Cyst Fibros*. 2015;14(1):16-25.
173. Li H, Salomon JJ, Sheppard DN, Mall MA, Galietta LJV. Bypassing CFTR dysfunction in cystic fibrosis with alternative pathways for anion transport. *Curr Opin Pharmacol*. 2017;34:91-97.
174. Milla CE, Ratjen F, Marigowda G, et al. Lumacaftor/Ivacaftor in patients aged 6-11 years with cystic fibrosis and homozygous for F508del-CFTR. *Am J Respir Crit Care Med*. 2017;195(7):912-920.
175. Rowe SM, Verkman AS. Cystic Fibrosis Transmembrane Regulator Correctors and Potentiators. *Cold Spring Harb Perspect Med*. 2013;3(7). doi:10.1101/cshperspect.a009761
176. Riccio E, Zanfardino M, Ferreri L, et al. Switch from enzyme replacement therapy to oral chaperone migalastat for treating fabry disease: real-life data. *Eur J Hum Genet*. 2020;28(12):1662-1668.
177. Vockley J, Andersson HC, Antshel KM, et al. Phenylalanine hydroxylase deficiency: diagnosis and management guideline. *Genet Med*. 2014;16(2):188-200.
178. René P, Lanfray D, Richard D, Bouvier M. Pharmacological chaperone action in humanized mouse models of MC4R-linked obesity. *JCI Insight*. 2021;6(4).
179. Ahmed CM, Dwyer BT, Romashko A, et al. SRD005825 acts as a pharmacologic chaperone of opsin and promotes survival of photoreceptors in an animal model of autosomal dominant retinitis pigmentosa. *Transl Vis Sci Technol*. 2019;8(6):30.
180. Li T, Sandberg MA, Pawlyk BS, et al. Effect of vitamin A supplementation on rhodopsin mutants threonine-17 → methionine and proline-347 → serine in transgenic mice and in cell cultures. *Proc Natl Acad Sci USA*. 1998;95(20):11933-11938.
181. Tam BM, Moritz OL. Dark rearing rescues P23H rhodopsin-induced retinal degeneration in a transgenic Xenopus laevis model of retinitis pigmentosa: a chromophore-dependent mechanism characterized by production of N-terminally truncated mutant rhodopsin. *J Neurosci*. 2007;27(34):9043-9053.
182. Janovick JA, Stewart MD, Jacob D, et al. Restoration of testis function in hypogonadotropic hypogonadal mice harboring a misfolded GnRHR mutant by pharmacoperone drug therapy. *Proc Natl Acad Sci U S A*. 2013;110(52):21030-21035.
183. Bernier V, Morello JP, Zarruk A, et al. Pharmacologic chaperones as a potential treatment for X-linked nephrogenic diabetes insipidus. *J Am Soc Nephrol*. 2006;17(1):232-243.
184. Kobayashi T, Sasaki S, Tomita N, et al. Synthesis and structure-activity relationships of 2-acylamino-4,6-diphenylpyridine derivatives as novel antagonists of GPR54. *Bioorg Med Chem*. 2010;18:3841-3859.
185. Kobayashi T, Sasaki S, Tomita N, et al. 2-acylamino-4,-6-diphenylpyridine derivatives as novel GPR54 antagonists with good brain exposure and in vivo efficacy for plasma LH level in male rats. *Bioorg Med Chem*. 2010;18:5157-5171.
186. Heitman LH, Ijzerman AP. G protein-coupled receptors of the hypothalamic-pituitary-gonadal axis: a case for Gnrh, LH, FSH, and GPR54 receptor ligands. *Med Res Rev*. 2008;28(6):975-1011.
187. Yang X, Lin G, Xia A, et al. Discovery of small molecule agonist of gonadotropin-releasing hormone receptor (GnRH1R). *J Chem Inf Model*. 2022;62(20):5009-5022.
188. Tello JA, Newton CL, Bouligand J, Guiochon-Mantel A, Millar RP, Young J. Congenital hypogonadotropic hypogonadism due to GnRH receptor mutations in three brothers reveal sites affecting conformation and coupling. *PLoS One*. 2012;7(6):e38456.
189. Tucci FC, Zhu YF, Struthers RS, et al. 3-[(2*R*)-Amino-2-phenylethyl]-1-(2,6-difluorobenzyl)-5-(2-fluoro-3-methoxyphenyl)-6-methylpyrimidin-2,4-dione (NBI 42902) as a potent and orally active antagonist of the human gonadotropin-releasing hormone receptor. Design, synthesis, and in vitro and in vivo characterization. *J Med Chem*. 2005;48(4):1169-1178.
190. Forfar R, Lu ZL. Role of the transmembrane domain 4/extracellular loop 2 junction of the human gonadotropin-releasing hormone receptor in ligand binding and receptor conformational selection. *J Biol Chem*. 2011;286(40):34617-34626.
191. Lu Z-L, Coetsee M, White CD, Millar RP. Structural determinants for ligand-receptor conformational selection in a peptide G protein-coupled receptor. *J Biol Chem*. 2007;282(24):17921-17929.
192. Leanos-Miranda A, Janovick JA, Conn PM. Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone receptor-mediated hypogonadotropic hypogonadism. *J Clin Endocrinol Metab*. 2002;87(10):4825-4828.
193. Maya-Núñez G, Janovick JA, Aguilar-Rojas A, et al. Biochemical mechanism of pathogenesis of human gonadotropin-releasing hormone receptor mutants $Thr^{104}Ile$ and $Tyr^{108}Cys$ associated with familial hypogonadotropic hypogonadism. *Mol Cell Endocrinol*. 2011;337(1–2):16-23.
194. Janovick JA, Patny A, Mosley R, et al. Molecular mechanism of action of pharmacoperone rescue of misrouted GPCR mutants: the GnRH receptor. *Mol Endocrinol*. 2009;23(2):157-168.
195. Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. *Endocr Rev*. 2004;25(2):235-275.
196. Betz SF, Reinhart GJ, Lio FM, Chen C, Struthers RS. Overlapping, nonidentical binding sites of different classes of nonpeptide antagonists for the human gonadotropin-releasing hormone receptor. *J Med Chem*. 2006;49(2):637-647.
197. Cui J, Smith RG, Mount GR, et al. Identification of Phe313 of the gonadotropin-releasing hormone (GnRH) receptor as a site critical for the binding of nonpeptide GnRH antagonists. *Mol Endocrinol*. 2000;14(5):671-681.
198. Nataraja SG, Yu HN, Palmer SS. Discovery and development of small molecule allosteric modulators of glycoprotein hormone receptors. *Front Endocrinol*. 2015;6:142.
199. Anderson RC, Newton CL, Anderson RA, Millar RP. Gonadotropins and their analogs: current and potential clinical applications. *Endocr Rev*. 2018;39(6):911-937.
200. van Koppen CJ, Zaman GJR, Timmers CM, et al. A signaling-selective, nanomolar potent allosteric low molecular weight agonist for the human luteinizing hormone receptor. *Naunyn Schmiedebergs Arch Pharmacol*. 2008;378:503-514.

201. Newton CL, Anderson RC, Kreuchwig A, Krause G, Katz AA, Millar RP. Rescue of function of mutant luteinising hormone receptors with deficiencies in cell surface expression, hormone binding, and hormone signalling. *Neuroendocrinology*. 2021;111(5):451-464.
202. Duan J, Xu P, Cheng X, et al. Structures of full-length glycoprotein hormone receptor signalling complexes. *Nature*. 2021;598(7882):688-692.
203. Gerrits M, Mannaerts B, Kramer H, Addo S, Hanssen R. First evidence of ovulation induced by oral LH agonists in healthy female volunteers of reproductive age. *J Clin Endocrinol Metab*. 2013;98(4):1558-1566.
204. Bonger KM, Hoogendoorn S, van Koppen CJ, Timmers CM, van der Marel GA, Overkleeft HS. Development of selective LH receptor agonists by heterodimerization with a FSH receptor antagonist. *ACS Med Chem Lett*. 2011;2(1):85-89.
205. Lin CC, Clouser C, Peegel H, Menon B, Menon KM. The extracellular domain of luteinizing hormone receptor dictates its efficiency of maturation. *Biochem Biophys Res Commun*. 2008;377(1):307-311.
206. Janovick JA, Maya-Núñez G, Ulloa-Aguirre A, et al. Increased plasma membrane expression of human follicle-stimulating hormone receptor by a small molecule thienopyr(im)idine. *Mol Cell Endocrinol*. 2009;298(1–2):84-88.
207. Nataraja S, Sriraman V, Palmer S. Allosteric regulation of the follicle-stimulating hormone receptor. *Endocrinology*. 2018;159(7):2704-2716.
208. Timmers CM, Loozen HJJ, inventors. Dihydrobenzoindazoles. Patent WO/2011/012674. 2011.
209. Hanyroup S, Anderson RC, Nataraja S, Yu HN, Millar RP, Newton CL. Rescue of cell surface expression and signaling of mutant follicle-stimulating hormone receptors. *Endocrinology*. 2021;162(12).
210. van Straten NC, Schoonus-Gerritsma GG, van Someren RG, et al. The first orally active low molecular weight agonists for the LH receptor: thienopyr(im)idines with therapeutic potential for ovulation induction. *Chembiochem*. 2002;3(10):1023-1026.
211. Jayasena CN, Comninos AN, Stefanopoulou E, et al. Neurokinin B administration induces hot flushes in women. *Sci Rep*. 2015;5:8466.
212. Zhang W-W, Wang Y, Chu Y-X. Tacr3/NK3R: beyond their roles in reproduction. *ACS Chem Neurosci*. 2020;11(19):2935-2943.
213. Malherbe P, Bissantz C, Marcuz A, et al. Me-talnetant and osanetant interact within overlapping but not identical binding pockets in the human tachykinin neurokinin 3 receptor transmembrane domains. *Mol Pharmacol*. 2008;73(6):1736-1750.
214. Sarau HM, Griswold DE, Potts W, et al. Nonpeptide tachykinin receptor antagonists: I. Pharmacological and pharmacokinetic characterization of SB 223412, a novel, potent and selective neurokinin-3 receptor antagonist. *J Pharmacol Exp Ther*. 1997;281(3):1303-1311.
215. Elliott JM, Carling RW, Chambers M, et al. N',2-diphenylquinoline-4-carbohydrazide based NK3 receptor antagonists. *Bioorg Med Chem Lett*. 2006;16(22):5748-5751.
216. Khan MIH, Sawyer BJ, Akins NS, le HV. A systematic review on the kappa opioid receptor and its ligands: new directions for the treatment of pain, anxiety, depression, and drug abuse. *Eur J Med Chem*. 2022;243:114785.
217. Chen Y, Chen C, Wang Y, Liu-Chen LY. Ligands regulate cell surface level of the human κ opioid receptor by activation-induced down-regulation and pharmacological chaperone-mediated enhancement: differential effects of nonpeptide and peptide agonists. *J Pharm Exp Ther*. 2006;319(2):765-775.
218. Petaja-Repo UE, Hogue M, Bhalla S, Laperrière A, Morello JP, Bouvier M. Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation. *EMBO J*. 2002;21(7):1628-1637.
219. Janovick JA, Brothers SP, Cornea A, et al. Refolding of misfolded mutant GPCR: post-translational pharmacoperone action in vitro. *Mol Cell Endocrinol*. 2007;272(1–2):77-85.
220. Ulloa-Aguirre A, Janovick JA, Miranda AL, Conn PM. G-protein-coupled receptor trafficking: understanding the chemical basis of health and disease. *ACS Chem Biol*. 2006;1(10):631-638.
221. Jaquette J, Segaloff DL. Temperature sensitivity of some mutants of the lutropin/choriogonadotropin receptor. *Endocrinology*. 1997;138(1):85-91.
222. Leidenheimer NJ, Ryder KG. Pharmacological chaperoning: a primer on mechanism and pharmacology. *Pharmacol Res*. 2014;83:10-19.
223. Smith E, Janovick JA, Bannister TD, et al. Rescue of mutant gonadotropin-releasing hormone receptor function independent of cognate receptor activity. *Sci Rep*. 2020;10(1):10579.
224. Janovick JA, Spicer TP, Smith E, et al. Receptor antagonism/agonism can be uncoupled from pharmacoperone activity. *Mol Cell Endocrinol*. 2016;434:176-185.
225. Feng X, Zhang M, Guan R, Segaloff DL. Heterodimerization between the lutropin and follitropin receptors is associated with an attenuation of hormone-dependent signaling. *Endocrinology*. 2013;154(10):3925-3930.
226. Leanos-Miranda A, Ulloa-Aguirre A, Ji TH, Janovick JA, Conn PM. Dominant-negative action of disease-causing gonadotropin-releasing hormone receptor (GnRHR) mutants: a trait that potentially coevolved with decreased plasma membrane expression of GnRHR in humans. *J Clin Endocrinol Metab*. 2003;88(7):3360-3367.
227. Ke R, Lok SIS, Singh K, Chow BKC, Janovjak H, Lee LTO. Formation of Kiss1R/GPER heterocomplexes negatively regulates Kiss1R-mediated signalling through limiting receptor cell surface expression. *J Mol Biol*. 2021;433(7):166843.
228. Cechova K, Lan C, Macik M, Barthes NPF, Jung M, Ulbrich MH. Kappa but not delta or mu opioid receptors form homodimers at low membrane densities. *Cell Mol Life Sci*. 2021;78(23):7557-7568.
229. Wang D, Sun X, Bohn LM, Sadée W. Opioid receptor homo- and heterodimerization in living cells by quantitative bioluminescence resonance energy transfer. *Mol Pharmacol*. 2005;67(6):2173-2184.
230. Jonas KC, Fanelli F, Huhtaniemi IT, Hanyaloglu AC. Single molecule analysis of functionally asymmetric G protein-coupled receptor (GPCR) oligomers reveals diverse spatial and structural assemblies. *J Biol Chem*. 2015;290(7):3875-3892.
231. Zarinan T, Perez-Solís MA, Maya-Núñez G, et al. Dominant negative effects of human follicle-stimulating hormone receptor expression-deficient mutants on wild-type receptor cell surface expression. Rescue of oligomerization-dependent defective receptor expression by using cognate decoys. *Mol Cell Endocrinol*. 2010;321(2):112-122.
232. Brothers SP, Cornea A, Janovick JA, Conn PM. Human loss-of-function gonadotropin-releasing hormone receptor mutants retain wild-type receptors in the endoplasmic reticulum: molecular basis of the dominant-negative effect. *Mol Endocrinol*. 2004;18(7):1787-1797.
233. Ballesteros JA, Weinstein H. Analysis and refinement of criteria for predicting the structure and relative orientations of transmembranal helical domains. *Biophys J*. 1992;62(1):107-109.

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