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G Protein-Coupled Receptors Involved in GnRH Regulation: Molecular Insights from Human Disease

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Abstract

In the past two decades, an increasing body of evidence has demonstrated that several G protein-coupled receptor (GPCR)-ligand pairs are critical for normal human reproductive development and function. Patients harboring genetic insults in either the receptors or their cognate ligands have presented with reproductive disorders characterized by varying degrees of GnRH deficiency. These disorders include idiopathic hypogonadotropic hypogonadism (IHH) and Kallmann Syndrome (KS). Conversely, mutations in some of these ligand-receptor pairs have been associated with accelerated reproductive maturation, manifested as central precocious puberty (CPP). To date, a series of elegant studies have characterized four GPCRs that play important roles in the neuroendocrine control of human reproductive development and function: GnRHR, KISS1R, PROKR2 and NK3R. Furthermore, these studies provide insights into the mechanisms by which mutations in these receptors give rise to reproductive disease phenotypes. This report will review mutations identified in GPCRs involved in the neuroendocrine control of the human reproductive axis with the aims of elucidating structure-function relationships of these GPCRs and identifying correlations between these structure-function relationships and the genotypic-phenotypic characterization of the patients.

Key terms

GPCR; hypogonadotropic hypogonadism; mutation

Introduction

Gonadotropin-releasing hormone (GnRH) deficiency is the underlying feature of several developmental and reproductive disorders including idiopathic hypogonadotropic hypogonadism (IHH) and Kallmann Syndrome (KS). Patients with IHH present with absent or delayed puberty with abnormally low gonadotropin and sex steroid levels, while patients with KS present with IHH associated with anosmia (loss of the sense of smell). Genetic studies of patients with GnRH deficiencies have vastly increased our understanding of the molecular mechanisms involved in GnRH regulation (for review, see Bianco and Kaiser,

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2009). During development, GnRH neurons migrate from the olfactory placode along the olfactory pathway to help guide them to their final destination in the hypothalamus. In the hypothalamus, GnRH is released from axon terminals in the median eminence into the hypophyseal portal circulation, where it acts at the level of the pituitary to stimulate gonadotropin release. To date, mutations have been identified in patients with GnRH deficiency in genes that play critical roles in both GnRH neuronal migration and GnRH secretion. The list of genes includes four G protein-coupled receptors (GPCRs) and their cognate ligands, implicating these GPCR-ligand pairs as important players in establishing and maintaining normal reproductive function in humans through their effects on GnRH neuronal migration and function. The genes encoding these GPCRs and their ligands are: *GNRH* and *GNRHR*, *KISS1* (encoding kisspeptin) and *KISS1R*, *PROKR2* (encoding prokineticin 2) and *PROKR2*, *TAC3* (encoding neurokinin B, or NKB) and *TACR3* (encoding NK3R). Although this review focuses on the GPCRs involved in GnRH regulation, it is important to note that mutations have been identified in the ligands as well, further underscoring the roles of these ligand-receptor pairs in GnRH regulation (for review see Bianco and Kaiser, 2009; Brioude et al., 2010).

GPCRs involved in GnRH regulation belong to the large rhodopsin superfamily of GPCRs that mediate the effects of many biological stimuli including peptide hormones and odorants (Takeda et al., 2002). These receptors share a common structure consisting of an amino terminus, a seven transmembrane (TM) domain connected by three extracellular and intracellular loops (ECL and ICL respectively) and a carboxy terminus. Biochemical and mutational analysis along with increasingly advanced high-resolution structural studies have identified the structure and role of individual domains in GPCR trafficking, activation and signaling (for review, see Baldwin, 1994; Wess 1998; Rosenbaum et al., 2009). In general, the amino terminus and the extracellular loops are involved in ligand recognition and binding although these domains may also play roles in GPCR signaling (for review, see Wess, 1998; Peeters et al., 2011); the TM domains are important for ligand binding and receptor trafficking (see Tan et al., 2004), while the intracellular loops and carboxy terminus are involved in GPCR coupling to G proteins and -arrestin for activation of signaling cascades (see Wess, 1998; Tikhonova and Costanzi, 2009) as well as in receptor internalization, recycling, desensitization and resensitization (for review, see Ferguson et al., 1998).

GPCRs are synthesized in the endoplasmic reticulum and then further modified in the Golgi before being transported to the cell surface (Bellotti et al., 1999; Brooks, 1999). Chaperone proteins interact with GPCRs to promote proper folding of GPCRs and facilitate the movement of GPCRs from the Golgi to the cell surface (Ellgaard, 1999; Hartl and Hartl-Hayer, 2002; Horwich, 2002). Upon ligand binding, GPCRs undergo conformational changes, couple to G proteins and activate downstream signal transduction pathways (for review, see Naor et al., 2000). Mutations in genes encoding GPCRs can therefore interfere with receptor expression, trafficking, ligand binding and activation of signaling cascades or other GPCR properties resulting in disease phenotypes.

In this review, we examined the structure-function relationships of *GNRHR*, *KISS1R*, *PROKR2* and *NK3R*, with an emphasis on the location and functional consequences of mutations identified in patients with GnRH dysregulation. While the three-dimensional crystal structure of these GPCRs have not yet been described, biochemical analyses and predicted models are useful to identify structural and functional domains in these GPCRs.

GNRHR

Prior to the cloning of the *GNRHR* gene, early studies demonstrated that the effects of GnRH were mediated through a receptor that was expressed at the cell surface and activated

the phospholipase C signaling pathway (see Conn et al., 1986, 1987). These studies provided evidence that GnRH acted through a GPCR; however, this was not confirmed until the cloning of the *GNRHR* gene by several groups (Kakar et al., 1992; Reinhart et al., 1992; Tsutsumi et al., 1992; Chi et al., 1993; Fan et al., 1994). The human *GNRHR* gene is localized to the long arm of chromosome 4 at position 21.2 and consists of 3 exons that encode a 328 amino acid protein (Kaiser et al., 1994). Two unique and interesting features of the human GnRHR are the lack of an intracellular carboxy terminal tail and the presence of a lysine residue at position 191 in the second ECL that modulates receptor conformation and expression. The absence of Lys191 in the rodent GnRHR confers increased cell surface expression or receptor stability in these animals (Arora et al., 1999). Deletion of Lys191 from human GnRHR increased receptor expression 4-fold, decreased EC₅₀ values by 5-fold for agonist-induced inositol phosphate (IP) production and significantly reduced the rate of internalization compared to wild-type human GnRHR (Arora et al., 1999). The cloning of the *GNRHR* gene led to additional insights into GnRHR trafficking and signaling (for review, see Kaiser et al., 1997; Armstrong et al., 2011).

Two separate groups initially reported loss-of-function mutations in the *GNRHR* gene in patients with IHH (de Roux et al., 1997; Layman et al., 1998). To date, at least 20 additional mutations in *GNRHR* have been identified in patients with sporadic and familial IHH, with an autosomal recessive pattern of inheritance and the majority of patients harboring compound heterozygous mutations (Table 1) (for review, see Bhagavath et al., 2005; Tao, 2006; Bedecarrats and Kaiser, 2007). These mutations are distributed throughout the various domains of the GnRHR, allowing for some interesting observations regarding structure–function correlations (Figure 1).

In 1997, de Roux and colleagues identified two mutations, Gln106Arg and Arg262Gln in ECL 1 and ICL 3 respectively, in a male proband and his sister who presented with incomplete IHH (de Roux et al., 1997). Both patients were compound heterozygotes, and responded to GnRH treatment. Binding studies showed that, consistent with known roles of GPCR extracellular loops in ligand binding, the Gln106Arg GnRHR mutation in ECL 1 resulted in reduced binding of a radiolabelled GnRH agonist (¹²⁵I-GnRH-A) compared to wild-type GnRHR, whereas the Arg262Gln GnRHR mutation in ICL 3 did not affect ¹²⁵I-GnRH-A binding. Both mutants had reduced GnRH-stimulated IP accumulation compared to wild-type GnRHR. This important finding demonstrated that a mutation in the ECL of the GnRHR impaired ligand binding leading to reduced GnRH-stimulated intracellular signal transduction, thereby likely contributing to the reproductive phenotype observed in these patients. On the other hand, the Arg262Gln GnRHR in ICL 3 had no effect on GnRH binding but had reduced ligand-stimulated IP accumulation compared to wild-type GnRHR (de Roux et al., 1997), presumably by interfering with G protein interactions with GnRHR, consistent with the role of intracellular loops in GPCR signaling. The Gln106Arg and Arg262Gln mutations are the most commonly identified mutations in GnRHR (for review, see Bedecarrats and Kaiser, 2007). While these two mutations result in partially impaired receptor function, a male patient homozygous for the Gln106Arg mutation was able to undergo spontaneous reversal of his reproductive phenotype (Pitteloud et al., 2001). Another female IHH patient also homozygous for the Gln106Arg mutation had a spontaneous pregnancy (Dewailly et al., 2002) and a third patient homozygous for Arg262Gln had a mild phenotype with delayed puberty (Lin et al., 2006). Similar to the patient described by de Roux and colleagues (1997), Beranova et al., (2001) reported a female IHH patient with compound heterozygous mutations in ECL 1 and ICL 3 (Gln106Arg and Leu266Arg). This patient also responded to GnRH treatment. However, in contrast to the Arg262Gln GnRHR mutant that bound GnRH but had impaired IP stimulation as described by de Roux (1997), functional studies of Leu266Arg GnRHR demonstrated that this mutation markedly impaired both binding and IP accumulation (Beranova et al., 2001; Bedecarrats et al., 2003).

This finding showed that although ICLs have generally been considered to have no direct role in ligand binding, mutations in ICLs can also impair ligand binding, likely by altering receptor structure. Taken together, the findings that exogenous GnRH administration can stimulate gonadotropin release in some IHH patients with GnRHR mutations, while others can undergo spontaneous reversal of their reproductive phenotype, demonstrate an elasticity in GnRH signaling which suggests that proper GnRH signaling may be crucial for progression through pubertal maturation, but that this defect can be overcome by exogenous GnRH or through some as yet unidentified mechanisms.

Recently, Millar and Newton (2010) developed a two-dimensional model of the GnRHR showing residues important for ligand binding (Asp98, Trp101, Asn102, Lys121, Asn212, Tyr290 and Asp302), as well as other residues that are either highly conserved across GPCRs or known to be involved in receptor structure, disulfide bond formation, glycosylation, receptor activation or signaling (Millar and Newton 2010). The residues involved in ligand binding lie within TM 2, 3, 5 and 6 domains, further demonstrating that these TM domains are important in forming the ligand binding pocket of the GnRHR. Although Gln106 itself is not a predicted binding site, its close proximity to residues that contribute to the predicted GnRHR ligand binding site could directly hinder GnRH binding. Interestingly, the Arg262Gln GnRHR occurs at a site predicted to be important for $G_{q/11}$ coupling to the GnRHR (Millar and Newton, 2010). This prediction that Arg262 is a site of $G_{q/11}$ coupling to the GnRHR provides an explanation for the decreased GnRH-stimulated IP accumulation that occurs *in vitro* with this GnRHR mutant.

Two other mutations, Thr104Ile and Tyr108Cys, in ECL 1, were identified in a female proband with complete IHH and her brother who presented with partial IHH (Antelli et al., 2006). Both patients were compound heterozygotes. The female proband responded to GnRH and estradiol treatment with progressive development of secondary sexual characteristics. However after treatment was stopped, a reevaluation of the pituitary-gonadal axis showed no significant differences in gonadotropin and sex steroid levels compared to pretreatment levels. On the other hand, her male sibling responded robustly to GnRH treatment with significantly elevated LH and testosterone levels both during and after treatment. The variability in phenotypes between these siblings with the same mutation suggests that gender or other genetic or environmental factors may influence the impact of mutations on phenotype. Recently, Maya-Nunez and colleagues (2011) characterized the effects of these two mutations on GnRHR expression and function. The Tyr108Cys mutant GnRHR had markedly reduced cell surface expression, ~98% reduction in 125 I-Buserelin (GnRH agonist) binding and ~97% reduction in IP production. Similarly, the Thr104Ile GnRHR mutant also had reduced cell surface expression with ~90% reduction in 125 I-Buserelin binding and ~70% reduction in IP production. Further studies demonstrated that both Thr104Ile and Tyr108Cys mutant receptor expression could be rescued by the pharmacoperone In3, a small nonpeptide GnRHR antagonist, or by deleting Lys191, suggesting that these mutations led to conformational changes in the receptor that impaired trafficking of the receptor to the cell surface (Maya-Nunez et al., 2011).

Another compound heterozygous mutation, Thr32Ile in the amino-terminus and Cys200Tyr in ECL2 of the GnRHR, was identified in a male patient who presented with IHH and gynecomastia and also responded to GnRH treatment. Functional studies demonstrated that both Thr32Ile and Cys200Tyr GnRHR mutations had no effect on receptor expression but failed to bind 125 I-Buserelin and had markedly reduced GnRH-stimulated IP accumulation (Beranova et al., 2001; Bedecarrats et al., 2003). The Cys200 residue in ECL 2 is thought to form a disulfide bond with Cys14 in the amino terminus to stabilize the receptor structure (Millar and Newton, 2010); therefore, a mutation of this residue may result in an altered receptor conformation that could impair binding.

While homozygous mutations are less frequently reported, a Cys279Tyr mutation in TM 6 has been reported (Beranova et al., 2001; Bedecarrats et al., 2003). The patient harboring this mutation failed to respond to GnRH treatment and functional studies demonstrated that this mutant failed to bind ^{125}I -Buserelin and had markedly reduced IP accumulation (Beranova et al., 2001; Bedecarrats et al., 2003). Based on the structure proposed by Millar and Newton (2010), Cys279Tyr in TM 6 is predicted to be important for the binding selectivity of GnRHR between GnRH I and GnRH II, suggesting that the substitution of a threonine for a cysteine could impair ^{125}I -Buserelin binding. In yet another study, a homozygous mutation, Arg139His, located in the conserved *DRS* motif at the junction of TM 3 and ICL 2 in the GnRHR, was identified in a female patient with IHH (Costa et al., 2001). This patient also did not respond to GnRH treatment. Similarly, the mutant Arg139His GnRHR failed to bind GnRH or activate signaling pathways. This Arg139 residue was predicted to be important in conformational structure of GnRHR and in GnRHR activation (Millar and Newton, 2010). Further evidence that GnRHR mutations affect receptor structure was provided by Conn and colleagues who demonstrated that In3, a GnRHR pharmacoperone, could rescue receptor expression and function of a number of GnRHR mutants, including Thr104Ile, Tyr108Cys, Thr32Ile, Cys200Tyr and Cys279Tyr (Janovick et al., 2002; Maya-Nunez et al., 2011).

Generally, from these reports, it appears that patients harboring homozygous mutations in *GNRHR* are less responsive to GnRH than compound heterozygotes, although it is not clear whether this difference in responsiveness is due to the homozygosity or to the fact these mutations occurred at residues that play key roles in receptor structure and function. It should be noted that this is not the case for all homozygous GnRHR mutations, as Topaloglu and colleagues (2006) also reported a homozygous mutation, Arg139Cys, in two sisters with normosmic HH (nHH). Functional studies showed that this GnRHR mutation impaired both ligand binding and receptor signaling, yet these patients responded to GnRH treatment. Compound heterozygous mutations are also frequently associated with partial IHH; there is variability in both the functionality of the mutant receptors and in the ability of the affected patients to respond to GnRH treatment, with imperfect genotype-phenotype correlation and imperfect correlation of *in vitro* and *in vivo* responses, suggesting that additional modifying factors may determine the severity of the phenotype in patients harboring GnRHR mutations.

Recently, Re and colleagues (2010) demonstrated that GnRH receptors are present at the nuclear membrane of GnRHR-expressing HTR-8/SVneo cells or HEK 293 cells. GnRH treatment of isolated nuclei from these cells induced a significant increase in histone H3 acetylation and H3 phosphorylation within 15 minutes of stimulation (Re et al., 2010). These important findings reveal a novel receptor signaling mechanism which may involve epigenetic regulation of other genes. More importantly, these findings indicate that functional studies investigating classical GPCR signaling cascades may not be sufficient in analyzing the effects of mutations on receptor function. Whether KISS1R, PROKR2 and NK3R are similarly localized to nuclear membranes is not known, although KISS1R is expressed both at the plasma membrane and intracellularly (Pampillo et al., 2009).

KISS1R

In 2003, two independent groups reported that mutations in *KISS1R* caused IHH in members of two consanguineous families through an autosomal recessive mode of inheritance (de Roux et al., 2003; Seminara et al., 2003). Since these reports, at least 12 mutations, including 8 missense mutations, have been identified in *KISS1R* (Figure 2); most of these mutations have been characterized to determine their effects on receptor expression and function (Table 1) (for review see, Cerrato and Seminara, 2007; Bianco and Kaiser, 2009). KISS1R and its ligand, kisspeptin, were originally implicated in cancer biology, as the

KISS1 gene was repressed in certain melanoma cell lines (Lee et al., 1996) and suppressed metastases of human melanomas and breast carcinomas (Lee and Welch 1997a; 1997b). More recently, both kisspeptin and *KISS1R* have been demonstrated to play critical roles in reproduction as kisspeptin is a potent stimulator of GnRH and gonadotropin release in primates and rodents (Gottsch et al., 2004; Matsui et al., 2004; Shahab et al., 2005). In addition knockout mouse models of both *KISS1* and *KISS1R* display an HH phenotype (Seminara et al., 2003; Funes et al., 2003). The human *KISS1R* gene, located on chromosome 19, has 5 exons and encodes a 398 amino acid protein. In 2003, de Roux and colleagues identified a homozygous 155 base pair deletion in 5 children with IHH from a consanguineous family. The proband and three male siblings presented with similar clinical features, with the proband having small testes and penis size with sparse pubic hair. A female sibling presented with partial IHH. All affected siblings had low sex steroid levels and had blunted responses to GnRH treatment (de Roux et al., 2003). In the same report, a Leu102Pro homozygous mutation in the *KISS1R* was identified in two other familial cases of IHH. The female proband who presented with primary amenorrhea and incomplete pubertal development was reported to harbor a homozygous Leu102Pro mutation in *KISS1R*. She had low estradiol levels with normal basal gonadotropin levels. She also had a sister with primary amenorrhea and a brother with delayed puberty. The proband was treated with estrogen and progestin followed by GnRH which ultimately resulted in two normal term pregnancies (Tenenbaum-Rakover et al., 2007). In a second family, the proband presented with micropenis and bilateral cryptorchidism and two sisters presented with primary amenorrhea. Functional studies of the Leu102Pro *KISS1R* mutation, which occurs in ECL 1, revealed that this mutation resulted in decreased cell surface expression, a 70% reduction in total binding, and more than 95% impairment in *KISS1*-stimulated IP accumulation compared to wild-type *KISS1R* (Tenenbaum-Rakover et al., 2007). These data suggest that this mutation in ECL 1 interferes with receptor trafficking. While another ECL mutation was identified in a male IHH patient harboring compound heterozygous mutations, Arg297Leu in ECL 3 along with Cys223Arg in TM 5, no functional studies were done to examine expression or binding; calcium mobilization assays demonstrated that the Cys223Arg mutation markedly reduced *KISS1*-stimulated calcium mobilization, whereas the Arg297Leu mutation had little effect, retaining 85% of the activity of wild-type *KISS1R* (Semple et al., 2005).

In the other seminal article on the role of *KISS1R* in GnRH deficiency, Seminara and colleagues (2003) described a homozygous mutation, Leu148Ser, in six affected offspring with IHH from a consanguineous family. Consistent with the role of ICL 2 in GPCRs, functional studies demonstrated that this mutation impairs the ability of *KISS1R* to activate IP signaling (Seminara et al., 2003) but does not affect *KISS1R* expression or ligand binding capabilities (Wacker et al., 2008). This mutation occurs at a leucine residue in ICL 2 that is highly conserved across species as well as in other GPCRs, and was predicted to serve as a site for G protein coupling in the GnRHR (Millar and Newton, 2010). Interestingly, co-immunoprecipitation studies investigating the mechanisms by which the Leu148Ser mutation impairs *KISS1R* function, demonstrated that the Leu148Ser mutant interacted with G proteins similar to wild-type *KISS1R*, but showed that the Leu148Ser mutation inhibited activation and dissociation of the G and G subunits (Wacker et al., 2008). These findings elucidate a complex signaling mechanism for GPCRs and illustrate that while mutations identified in GPCRs can affect receptor coupling to G proteins, mutations can also alter interactions between the individual G protein subunits. Seminara and colleagues (2003) also reported a male patient with sporadic IHH harboring an R331X and X399R compound heterozygous mutation. These types of mutations have been shown to be targeted to nonsense-mediated or nonstop decay (Maquat and Carmichael, 2001; Frischmeyer et al., 2002; van Hoof et al., 2002). Quantitative real-time RT-PCR confirmed that these two mutations resulted in markedly reduced mRNA levels in lymphoblasts from the affected

patient, with the R331X and X399R mutant alleles having approximately 18% and 2.5% of normal levels and suggests that these mutations resulted in reduced receptor expression in the affected individual.

Recently, a homozygous mutation in TM 6 of KISS1R, Phe272Ser, was identified in six affected patients, 5 males and 1 female, from two consanguineous families (Nimri et al., 2011). At infancy, the males presented with cryptorchidism and micropenis, and no subsequent pubertal development was detected. At age 18, the female presented with impuberism and primary amenorrhea. Repeated GnRH stimulation failed to stimulate gonadotropin levels. Functional studies for this mutant KISS1R demonstrated that the Phe272Ser mutation led to reduced cell surface expression of the mutant KISS1R and impaired kisspeptin-induced IP signaling, compared to wild-type KISS1R. TM domains are important for receptor trafficking; therefore, the reduced cell surface mutant KISS1R expression may be due to trafficking defects of the receptor.

Although there are relatively few *KISS1R* mutations reported in the literature, more homozygous mutations than heterozygous or compound heterozygous mutations have been reported (Table 1). Interestingly, of the mutations that have been tested to date, only those identified in the homozygous or compound heterozygous state in patients have been demonstrated to cause deleterious effects on receptor function. It should be noted that the effect of mutations in *KISS1R* on other signaling pathways activated by KISS1R have not been widely examined and whether heterozygous KISS1R mutations affect other signaling cascades remain to be determined. Recent studies have demonstrated a key role for -arrestin-2 in activating kisspeptin-induced ERK phosphorylation (Pampillo et al., 2009; Szereszewski et al., 2010). -arrestins have been implicated in GPCR signaling cascades for some time (see Wess, 1998; Tikhonova and Costanzi, 2009); however, this finding by Babwah and colleagues (2009, 2010) demonstrates the importance of comprehensively analyzing a spectrum of signaling pathways to assess the impact of mutations on receptor function. It may therefore be important to examine the effects of heterozygous KISS1R mutations on ERK phosphorylation and perhaps even other signaling pathways to better understand whether these types of mutations affect receptor signaling. Nevertheless, KISS1R loss-of-function mutations display clear structure-function relationships with ECL and TM mutations impairing receptor expression associated with decreased binding and subsequent loss of receptor signaling and ICL mutations affecting receptor signaling but having little or no effect on ligand binding.

In contrast to the increasing number of reports identifying loss-of-function mutations in KISS1R and other GPCRs involved in GnRH regulation, to date, only one gain-of-function mutation has been reported in a patient with central precocious puberty (CPP) (Teles et al., 2008). This study reported a heterozygous mutation (Arg386Pro) in *KISS1R* in a young girl with CPP. The 8 year old patient presented with advanced Tanner stage 4 breast development and Tanner stage 2 pubic hair. Serum estradiol levels were elevated together with nonsuppressed LH and FSH levels. An injection of a GnRH agonist stimulated LH levels, consistent with central activation of the reproductive axis. Functional studies demonstrated that this mutation had no effect on KISS1R expression, ligand binding affinity, or acute activation of signal transduction pathways; however, kisspeptin treatment induced prolonged activation of intracellular signaling pathways, including stimulation of IP accumulation and ERK phosphorylation, compared to wild-type receptors. This apparent reduced rate of desensitization may increase the stimulatory effects of kisspeptin on GnRH secretion, thus accelerating the maturation of the reproductive axis (Teles et al., 2008). Further studies to elucidate the mechanisms of these effects showed that the Arg386Pro KISS1R mutation prolongs responsiveness to kisspeptin by decreasing KISS1R degradation after ligand-induced internalization, resulting in increased recycling of the Arg386Pro

KISS1R back to the plasma membrane compared to the wild-type receptor (Bianco et al., 2011). Similarly, a Pro74Ser *KISS1* mutation was identified in a patient with CPP. The patient presented at 17 months of age with enlarged penis and testes and Tanner stage 3 pubic hair; hormonal evaluation demonstrated a high LH and testosterone levels. Functional analyses indicated that Pro74Ser kisspeptin was more resistant to degradation, suggesting greater kisspeptin bioavailability and a potential role in the precocious puberty phenotype through a mechanism analogous to that described for Arg386Pro KISS1R (Silveira et al., 2010).

PROKR2

The PROK2/PROKR2 system regulates various biological processes including intestinal contraction, circadian rhythms, vascular function as well as development of the olfactory and reproductive systems (Schweitz et al., 1999; Li et al., 2001; Cheng et al., 2002; Matsumoto et al., 2006; Dode et al., 2006; Prosser et al., 2007; Pitteloud et al., 2007). This ligand-receptor pair became a candidate gene for genetic analysis in patients with KS as knock-out mouse models of both *Prok2* and *Prokr2* exhibited defects in both olfactory bulb development and GnRH neuronal migration (Matsumoto et al., 2006; Pitteloud et al., 2007).

In 2006, Dode and colleagues first reported that mutations in the *PROKR2* and *PROK2* genes caused disease phenotypes in patients with KS (Dode et al., 2006). To date, at least 26 missense mutations in *PROKR2* have been reported in patients with IHH or KS (for review, see Martin et al., 2011). Interestingly, while the majority of mutations in *GNRHR* and *KISS1R* in patients with GnRH deficiency are found in either compound heterozygous or homozygous states, many *PROKR2* mutations reported in patients with KS or IHH are present in the heterozygous state (Table 1). While functional studies demonstrated that several *PROKR2* mutations impair receptor activity, whether heterozygous mutations by themselves are sufficient to cause GnRH deficiency in these patients is a topic of much debate. Moreover, several of these mutations have been identified in unaffected individuals (Dode et al., 2006; Abreu et al., 2008; Sykiotis et al., 2010). It appears that mutations in *PROKR2* may have several modes of inheritance including autosomal recessive or autosomal dominant modes with incomplete penetrance as patients are observed with varying phenotypes and varying degrees of GnRH deficiency. In trying to assess the mechanisms by which heterozygous mutations in *PROKR2* may cause GnRH deficiency, several investigators tested *PROKR2* mutants for dominant negative effects on wild-type *PROKR2*. To date, no *PROKR2* mutants have been demonstrated to have dominant negative effects, indicting digenic or oligogenic modes of inheritance or other modifying factors in the mechanisms through which heterozygous *PROKR2* mutations may result in disease phenotypes (Monnier et al., 2009; Sykiotis et al., 2010). It should be noted that the cause of KS in the majority of patients remains unknown, suggesting that there are other genes yet to be identified that could contribute (Cole et al., 2008; Dode and Hardelin, 2008; Sarfati et al., 2010; Sykiotis et al., 2010). Interestingly, several *PROKR2* mutations have been identified in patients harboring mutations in various other genes associated with GnRH deficiency including *FGFR1*, *KAL1* and *PROK2* (Cole et al., 2008; Monnier et al., 2009; Sarfati et al., 2010; Sykiotis et al., 2010), further supporting the hypothesis that digenic modes of inheritance may underlie disease phenotypes in patients harboring *PROKR2* mutations.

Dode and colleagues (2006) identified nine missense mutations and one frameshift mutation in *PROKR2* in patients with KS. The nine missense mutations were found in homozygous, compound heterozygous and heterozygous states and were localized across multiple domains of *PROKR2*: Gln210Arg in ECL 2, Leu173Arg, Trp178Ser, Pro290Ser, Met323Ile, and Val331Met in TM domains, and Arg85His, Arg164Gln and Arg268Cys in ICLs (Figure 3). Two mutations (Arg268C and Val331Met) were also identified in the control group suggesting that either these mutations are not disease causing or some additional

mechanisms contribute to disease phenotypes in the patients. Functional studies were conducted to assess whether these mutations impaired receptor function (Monnier et al., 2009). The Gln210Arg mutation in ECL2, found in both heterozygous and compound heterozygous states, had no effect on PROKR2 expression, but severely impacted ligand binding activity consistent with the role of ECLs, which subsequently impaired ligand-induced intracellular release of calcium. Three of the five TM mutations, Leu173Arg, Trp178Ser and Pro290Ser, had significant decreases in cell surface expression, ligand binding, and receptor signaling compared to wild-type PROKR2. The Trp178Ser and Pro290Ser PROKR2 mutants were identified in the heterozygous state while the Leu173Arg PROKR2 mutant was identified in heterozygous and homozygous states as well as in a compound heterozygous state with Gln210Arg. Interestingly both Trp178Ser and Pro290Ser, identified in the heterozygous state, had the most pronounced effects on receptor function. Three ICL mutations (Arg85His, Arg164Gln and Arg268Cys, were also identified by Dode and colleagues (2006). Several additional intracellular mutations have been reported in GnRH-deficient patients with a large number occurring at ICL arginine residues (see Abreu et al., 2008; Cole et al., 2008; Sykiotis et al., 2010). Using the GnRHR as a model (Millar and Newton, 2010), most ICL arginine residues function as important sites for G protein coupling or activation. It is likely that mutations at these sites are more likely to be disease-causing by impairing receptor signaling. Other potential explanations for this observation may be that arginine residues in PROKR2 are more susceptible to compromise, leading to the high incidence of mutations at these sites. Functional studies of these ICL mutations demonstrated that while Arg164Gln and Arg268Cys had little effect if any, on receptor expression Arg85His caused a 40% decrease in receptor expression. Both Arg85His and Arg164Gln markedly impaired receptor signaling, suggesting that mutations in intracellular domains can affect receptor signaling and can also have effects on receptor expression (Dode et al., 2006).

Similarly, Cole et al. (2008) identified six novel mutations in PROKR2 as well as four previously identified mutations in a cohort of 324 patients with GnRH deficiency. All patients were heterozygous for mutations in PROKR2, with one patient harboring a mutation in both the receptor (Val115Met) as well as the ligand. Two patients with IHH, each harboring an ICL mutation in the heterozygous state, Arg85Cys or Arg248Gln, had partial puberty suggesting partial GnRH deficiency; the patient with the Arg248Gln PROKR2 mutation subsequently had reversal of hypogonadism after discontinuation of testosterone treatment, achieving normal testosterone levels and fertility. To analyze the effects of these mutations on receptor function, Cole et al. (2008) examined activation of the mitogen-activated protein kinase (MAPK) and calcium mobilization pathways. Functional studies of Arg85Cys and Arg248Gln PROKR2 demonstrated little effect on receptor expression or MAPK signaling; however, calcium mobilization was moderately impaired. Three of the other novel mutations reported (Tyr113His, Val115Met, Ser188Leu) all had severe effects on both MAPK signaling and calcium mobilization. The functional studies of mutations in PROKR2 identified in patients GnRH deficiency clearly demonstrated that these mutations affect receptor expression and function and may contribute to the disease phenotype.

TACR3

Neurokinin B (NKB) is a member of the tachykinin family which includes substance P, neurokinin A, and neuropeptide K. It has been shown to have modulatory functions in several regions of the brain, including excitatory roles in synaptic transmission in pyramidal neurons of the medial prefrontal cortex (Simmons et al., 2008). NKB binds to its G protein-coupled receptor, NK3R, and activates various signaling cascades including IP and PKC signaling pathways (Khawaja and Rogers, 1996). NK3R can also couple to G_s and G_i resulting in activation or inhibition of adenylyl cyclase (Khawaja and Rogers, 1996).

Previously, little was known about the role of NKB in GnRH regulation, although several studies implicated NKB in the changes in GnRH and gonadotropins in postmenopausal women (for review, see Rance, 2009).

Recently, genome-wide single nucleotide polymorphism analysis of nine consanguineous families led to the identification of two homozygous mutations, Gln93Asp and Pro353Ser, in the *TACR3* gene encoding NK3R in three families. Six individuals, 2 males and 4 females, were affected from these families. All females lacked spontaneous breast development and 3 females were also reported to present with primary amenorrhea. The males presented with small penises and 1 ml testicular volumes. All patients had abnormally low levels of LH but responded robustly to a GnRH stimulation test with increases in LH levels. Functional studies demonstrated that these two mutations in the TM domains impaired receptor signaling (Topaloglu et al., 2009). To date, at least six other NK3R missense mutations have been reported in patients with IHH in various domains of NK3R (Figure 4), implicating this receptor in GnRH regulation (Table 1) (Guran et al., 2009; Gianetti et al., 2010).

In the study by Gianetti and colleagues (2010), five novel missense mutations were identified in patients with IHH. Of the five mutations, two were identified in the homozygous state and three were in the heterozygous state. One homozygous mutation, Y256H in TM 5, was in a male patient who presented with small phallus, 5ml testes volume and low testosterone levels. Functional studies showed that this mutation resulted in a marked decrease in NKB-induced IP accumulation compared to wild-type NK3R. Surprisingly, after testosterone treatment, this patient maintained pulses of LH with normal testosterone levels, indicating that patients with deleterious receptor mutations can overcome functional defects with phenotypic reversal. Another patient was identified with a heterozygous mutation, Arg295Ser, in ECL 3 of the NK3R, which also showed a marked decrease in IP signaling. While no comprehensive two-dimensional models are available for NK3R, the Arg295Ser mutation located in ICL 3 may be important for G protein coupling to NK3R, similar to arginine residues in ICL 3 of the GnRHR (Millar and Newton, 2010). Similarly, the lone tyrosine residue (Tyr211) in TM 5 of the GnRHR is involved in receptor activation, suggesting that the corresponding Tyr256His NK3R mutation in the same domain could serve the same function. The use of the I-Tasser bioinformatics program predicts that Tyr256 and Tyr315 located in TM 5 and TM6 may also be NK3R binding sites for NKB (Roy et al., 2010; Zhang, 2009) and therefore it is anticipated that mutations at these sites could reduce ligand binding affinity and thereby impair receptor function. Further work is required to assess whether these mutations affect receptor expression and ligand binding.

Concluding Remarks

It is evident that mutations in particular domains of receptors are at least in part predictive of the manner in which receptor expression or function is impaired. Although the crystal structures for these GPCRs have not yet been determined, prediction programs, mutational and biochemical analysis as well as comparison to other GPCRs with known structures highlight residues that may be critical for receptor structure and function. In this review, we have examined structure-function relationships using mutations in various domains of the GPCRs identified in patients with disorders of GnRH regulation to elucidate the functional roles of these domains and their amino acid residues. Studies of mutations identified in GnRHR, KISS1R, PROKR2 and NK3R have identified defects in receptor expression and function consistent with the known roles of the domains in which the mutations occurred, but have also provided new insights into GPCR biology. Proposed models by Zhou et al. (1994) as well as Millar and Newton (2010) have helped immensely in understanding the

structure-function relationships for GnRHR and have contributed to our understanding of these relationships in other GPCRs. While definitive structural information will await the elucidation of crystal structures of these receptors, continued functional analyses will further advance our understanding of structure-function relationships for these GPCRs involved in the neuroendocrine control of human reproductive function.

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Figure 1.
Missense mutations in GnRHR.

Figure 2.
Missense mutations in KISS1R.

Figure 3.
Missense mutations in PROKR2.

Figure 4.
Missense mutations in NK3R.

Table 1

Genotypes and phenotypes of patients with GnRH deficiency.

Mutation	Type	Phenotype	Exon	GPCR domain	Expression	Signaling	Binding	Reference
GnRHR								
Met1Thr	compound heterozygous	nHH	1	N-terminal	NT	NT	NT	Sykiotis et al., 2010
Asn10Lys	heterozygous	nHH	1	N-terminal	=			Costa et al., 2001
Gln11Lys	heterozygous	nHH	1	N-terminal				Meysing et al., 2004
Thr32Ile	compound heterozygous (Cys200Tyr)	nHH	1	N-terminal			Absent	Beranova et al., 2001; Bodecarrats et al., 2003
Glu90Lys	homozygous	nHH	1	TM2		Absent	Absent	Söderlund et al., 2001
Pro96Ser	compound heterozygous	nHH	1	TM2	NT	NT	NT	Sykiotis et al., 2010
Thr104Ile	compound heterozygous	nHH	1	ECL1		NT	NT	Antelli et al., 2006
Gln106Arg	compound heterozygous (Asn10Lys; Ala171Thr; Ser217Arg; Leu266Arg)	nHH	1	ECL1				De Roux et al., 1997; Costa et al., 2001
Tyr108Cys	compound heterozygous	nHH	1	ECL1		NT	NT	Antelli et al., 2006
Ala129Asp	compound heterozygous	nHH	1	TM3		Absent	Absent	Caron et al., 1999
Arg139His	homozygous, compound heterozygous	nHH	1	TM3		Absent	Absent	Costa et al., 2001
Arg139Cys	homozygous	nHH	1	TM3				Topaloglu et al., 2006
Pro146Ser	compound heterozygous	nHH			NT	NT	NT	Sykiotis et al., 2010
Ser168Arg	homozygous	nHH	1	TM4	=	Absent	Absent	Pralong et al., 1999
Ala171Thr	compound heterozygous (Gln106Arg)	nHH	1	TM4	=	Absent	Absent	Karges et al., 2003
Ser217Arg	compound heterozygous (Arg262Gln; Gln106Arg)	nHH	2	TM5		Absent	Absent	De Roux et al., 1999
Arg262Gln	compound heterozygous (Ser217Arg; Gln106Arg)	nHH	3	ICL3	=			De Roux et al., 1997; Caron et al., 1999
Leu266Arg	compound heterozygous (Gln106Arg)	nHH	3	ICL3	=	Absent	Absent	Beranova et al., 2001; Bodecarrats et al., 2003
Cys279Tyr	homozygous	nHH	3	TM6	=	Absent	Absent	Beranova et al., 2001; Bodecarrats et al., 2003
Tyr284Cys		nHH	3	TM6	=			Leaños-Miranda et al., 2005
Pro320Leu	compound heterozygous	nHH	3	TM7		Absent	Absent	Meysing et al., 2004
KISSIR								
Leu102Pro	homozygous	nHH	2	ECL1	NT			Tenenbaum-Rakover et al., 2007

Mutation	Type	Phenotype	Exon	GPCR domain	Expression	Signalin g	Binding	Reference
Leu148Ser	homozygous	nHH	3	ICL2	=		=	Seminara et al., 2003 Wacker et al., 2008
Cys223Arg	compound heterozygous	nHH	4	TM5	NT		NT	Semple et al, 2005
Glu252Gln	heterozygous	nHH	5	ICL3	NT	=	=	Teles et al., 2010
Phe272Ser	comozygous	nHH	5	TM6	(cell surface-IP)		NT	Nimri et al., 2011
Arg297Leu	compound heterozygous (C223R)	nHH	5	ECL3	NT		NT	Semple et al., 2005
His360Leu	heterozygous	nHH	5	C-terminal	NT	NT	NT	Cerrato et al., 2006
PROKR2								
Ala51Thr	heterozygous	HH	1	N-terminal	Not tested. PolyPhen-2 and Sift prediction: benign			Sykotiis et al., 2010
Met64Val	heterozygous	HH	1	TM1	Not tested PolyPhen-2 and Sift prediction: benign			Sykotiis et al., 2010
Arg80Cys	heterozygous	KS,	1	ICL1	Not tested			Abreu et al., 2008
Arg85His	Heterozygous, homozygous	KS, nHH, delayed puberty	1	ICL1	=			Dode et al., 2006 Monnier et al, 2009 Sarfati et al., 2010
Arg85Cys	Heterozygous, homozygous	KS and nHH	1	ICL1	=			Dode et al., 2006 Monnier et al, 2009 Sarfati et al., 2010 Cole et al., 2008.
Arg85Leu	heterozygous	KS	1	ICL1	Not tested			Sarfati et al., 2010
Arg85Gly	heterozygous	KS	1	ICL1	Not tested			Sarfati et al., 2010
Met111Arg	heterozygous	HH		ECL1	Not tested PolyPhen-2 and Sift prediction: damaging			Sykotiis et al., 2010
Tyr113His	heterozygous	KS	1	ECL1			NT	Cole et al., 2008.
Val115Met	heterozygous	KS	1	ECL1				Cole et al., 2008. Sykotiis et al., 2010
Arg164Gln	heterozygous	KS	2	ICL2	or =		=	Cole et al., 2008. Monnier et al, 2009
Leu173Arg	Homozygous, heterozygous compound heterozygous	KS, nHH	2	TM4 (close to ICL2)	or =			Cole et al., 2008. Monnier et al, 2009
Ser188Leu	heterozygous	KS	2	TM4			NT	Cole et al., 2008.
Ser202Gly	heterozygous	HH	2	ECL2	Not tested PolyPhen-2 and Sift prediction: benign			Sykotiis et al., 2010
Gln210Arg	heterozygous compound heterozygous (Arg173Leu)	KS	2	ECL2				Monnier et al, 2009 Sykotiis et al., 2010
Arg248Gln	heterozygous	nHH	2	ICL3	=		NT	Cole et al., 2008.

Mutation	Type	Phenotype	Exon	GPCR domain	Expression	Signalin g	Binding	Reference
Trp251Leu	heterozygous	KS	2	ICL3	Not tested			Sarfati et al., 2010
Thr260Met	heterozygous	HH	2	ICL3	Not tested PolyPhen-2 and Sift prediction: damaging			Sykitis et al., 2010
Arg268Cys	heterozygous	KS	2	ICL3	=			Monnier et al., 2009
Arg270Cys	heterozygous	HH	2	ICL3	Not tested PolyPhen-2 and Sift prediction: damaging			Sykitis et al., 2010
Val274Asp	homozygous	KS reversible	2	ICL3/TM6	Not tested			Sinisi et al., 2008
Pro290Ser	homozygous heterozygous	KS	2		=			Monnier et al., 2009
Met323Ile	compound heterozygous	KS	2	TM7	=	=	=	Monnier et al., 2009
Val331Met	heterozygous	KS	2	C-terminal	=			Monnier et al., 2009
Arg353Trp	heterozygous	KS	2	C-terminal	=		NT	Cole et al., 2008.
TACR3								
Gln18Asp	heterozygous	nHH	1	N-terminal	NT	=	NT	Gianetti et al., 2010
Gln93Asp	homozygous	nHH	1	TM1	NT		NT	Topaloglu et al., , 2009
His148Leu	homozygous	nHH	1	ECL1	NT		NT	Guran et al., 2009
Ile249Val	heterozygous	nHH	3	TM5	NT	=	NT	Gianetti et al., 2010
Tyr256His	homozygous	nHH	3	TM5	NT		NT	Gianetti et al., 2010
Arg295Ser	heterozygous	nHH	3	ICL3	NT		NT	Gianetti et al., 2010
Tyr315Cys	homozygous	nHH	4	TM6	NT		NT	Gianetti et al., 2010
Pro353Ser	homozygous	nHH	4	TM6	NT		NT	Topaloglu et al., , 2009