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A needle in a haystack: Mutations in *GNRH1* as a rare cause of isolated GnRH deficiency

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Abstract

GNRH1, the human gene that gives rise to GnRH, has long been an obvious candidate gene for idiopathic hypogonadotropic hypogonadism, particularly because the *hpg* mouse, a mouse model of isolated hypogonadotropic hypogonadism, carries a deletion that disrupts *Gnrh1*. In 2009, 25 years after the sequence of human *GNRH1* was initially determined, two groups independently reported homozygous frameshift mutations in *GNRH1* in patients with idiopathic hypogonadotropic hypogonadism. In two additional families, heterozygous *GNRH1* mutations segregated with idiopathic hypogonadotropic hypogonadism. In the first family, the mutation occurred alone in four female subjects, whereas in the second it co-existed with a mutation in *NROB1/DAX1* to cause delayed puberty in two female subjects. While hemizygous mutations the X-linked *NROB1* are a well-known cause of hypogonadotropic hypogonadism and adrenal hypoplasia in male patients, heterozygous female carriers are generally asymptomatic. Thus, mutations in *GNRH1* have been associated with both mild and severe forms of GnRH deficiency, and may work in combination with other gene mutations to produce GnRH-deficient phenotypes.

Keywords

Gonadotropin-releasing hormone (GnRH); luteinizing-hormone releasing hormone (LHRH, LRH); human genetics; gonadotropin-releasing hormone; *hypogonadal* (*hpg*) mouse; hypogonadotropic hypogonadism

1. Cloning of human *GNRH1*

In 1971, the groups of Guillemin and Schally independently purified mammalian GnRH and determined the amino-acid sequence of this decapeptide hormone, work for which the Nobel prize was awarded in 1977 (Figure 1; Amoss et al., 1971, Schally et al., 1971). In addition to opening many lines of physiological investigation, this discovery paved the way for the cloning and sequencing of the *GNRH1* gene and the determination of the primary structure

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of the GnRH precursor peptide in 1984 (Seeburg and Adelman, 1984). The GnRH preprohormone is a 92-amino acid peptide with an N-terminal signal sequence, followed by the GnRH decapeptide, a 3-amino acid cleavage site, then a 56-amino acid fragment called the GnRH-associated peptide (GAP, discussed further below). With the gene sequence of *GNRH1* determined, investigators could now use human genetic approaches to study the role of *GNRH1* in reproductive disorders.

2. Biological precedent set by the *hpg* mouse

A firm rationale for searching for *GNRH1* mutations in patients with IHH came from the *hpg* mouse, the first animal model of hypogonadotropic hypogonadism (Cattanach et al., 1977). The *hpg* mutation arose spontaneously at the Medical Research Council mouse facility at Harwell, UK, and homozygous *hpg* mice had hypoplastic genitalia and gonads and low circulating sex steroids and gonadotropins. Direct measurements of hypothalamic GnRH content demonstrated that *hpg* mice lacked GnRH. Subsequent experiments showed that *hpg* mice could respond to exogenous GnRH (Fink et al., 1982), demonstrating that pituitary and downstream functions remained intact and placing the defect in the *hpg* mouse at the level of GnRH neurons or above.

Subsequently, in a remarkable series of experiments, Krieger et al. (1982) transplanted tissue from the preoptic area of fetal mice, a region rich in GnRH neurons, into the third ventricle of adult *hpg* mice. The transplanted GnRH neurons sent axonal projections to the median eminence, and this resulted in rescue of gonadotropin secretion and reproductive function. This result suggested that the defect lay within the GnRH neurons themselves rather than in pathways lying further upstream.

Gnrh1 was therefore the most logical candidate gene for the cause of the isolated GnRH deficiency of *hpg* mice. Mason et al. (1986) tested this possibility by first cloning and sequencing the *Gnrh1* gene from wild-type mice. They then examined the gene structure of *Gnrh1* in *hpg* mice and identified a deletion affecting the distal half of the gene, with one breakpoint lying between the 2nd and 3rd exons of *Gnrh1* and the other breakpoint 41.7 kb downstream. This deletion leaves intact the proximal half of the gene, which encodes the N-terminal 45 amino acids of the GnRH preprohormone and includes the GnRH decapeptide. This truncated *Gnrh1* gene was able to drive normal transcription, as *Gnrh1* transcript was detectable in the hypothalamus of *hpg* mice, but not normal translation, as no GnRH immunoreactivity was detected.

The molecular mechanism for this translational block was determined by Son et al. (2005). The first intron of *Gnrh1* is variably spliced, and the splicing of this intron is required for translation to occur. The splicing of the first *Gnrh1* intron is dependent on sequences in the 3rd and 4th exons of *Gnrh1*, which are deleted by the *hpg* mutation. Thus, in *hpg* mice the splicing of intron 1 does not occur and the *Gnrh1* transcript is not translated.

Given the large size of the deletion, it was possible that genes other than *Gnrh1* could contribute to the phenotype of the *hpg* mice. To address this issue, Mason et al. (1986) used a transgene containing *Gnrh1* to rescue the hypogonadotropic hypogonadism of *hpg* mice. This notable experiment, regarded as the first instance of gene therapy in mammals, demonstrated that the reproductive phenotype of *hpg* mice was primarily if not entirely due to loss of *Gnrh1* function.

3. The search for *GNRH1* mutations in IHH

With *GNRH1* being such an obvious candidate gene for GnRH deficiency, several groups attempted to identify *GNRH1* mutations in GnRH-deficient patients using two general

approaches. The first was to screen for *GNRH1* deletions by Southern blotting (which could also detect restriction fragment length polymorphisms) or multiplex ligation-dependent polymerase amplification (MLPA). To date, the literature has reported results for over 261 patients screened by either method, and no deletions of *GNRH1* were found (Table 1; Layman et al., 1992; Nakayama et al., 1990; Pedersen-White et al., 2008; Trarbach et al., 2010; Weiss et al., 1989).

The second approach was to sequence *GNRH1*. Initial studies in small numbers of patients identified a single variant, W16S, that was also found in unaffected family members and subsequently shown to be a common polymorphism (Nakayama et al., 1990; Weiss et al., 1991). No other variants were found, and thus these early studies demonstrated that mutations in *GNRH1* were not a common cause of GnRH deficiency.

In the years following these initial studies, investigators continued to search for *GNRH1* mutations though few of these efforts were reported in the literature (Bo-Abbas et al., 2003). Our group screened hundreds of patients with isolated GnRH deficiency for unambiguously pathogenic mutations in *GNRH1* but met with no success. The inability to find mutations in such a promising candidate gene was discouraging and led to speculation about possible reasons for why *GNRH1* mutations were not found.

One possible explanation was that *GNRH1* may act redundantly with one or more other genes, so that loss of *GNRH1* function would be compensated for by the activity of these other genes. Indeed, humans have a second GnRH gene, *GNRH2* (White et al., 1998). However, the hypogonadotropic hypogonadism of *hpg* mice demonstrated that loss of *Gnrh1* alone is sufficient to produce a severe reproductive phenotype (Cattanach et al., 1977).

Another potential explanation for the inability to find *GNRH1* mutations was that GnRH may have essential functions outside of stimulation of gonadotropin secretion, and that mutations in *GNRH1* would therefore be lethal. For instance, GnRH is expressed in the placenta (Gibbons et al., 1975). If *GNRH1* mutations were to disrupt placental function, this phenomenon could lead to miscarriage or fetal demise early in gestation, well before any reproductive phenotype could be observed. Here, the *hpg* mouse again proved to be informative; homozygous *hpg* mice were fully viable and were born in expected Mendelian ratios (Cattanach et al., 1977). Thus, at least in the mouse, mutation of *Gnrh1* did not cause obvious phenotypes outside of hypogonadotropic hypogonadism. Human investigators therefore remained undaunted and persisted in the search for mutations in *GNRH1*.

3.1 At last, homozygous mutations in *GNRH1*

In 2009, Bouligand et al. screened ~150 probands with normosmic GnRH deficiency and identified one proband with a homozygous frameshift mutation in *GNRH1* (Bouligand et al., 2009; Bouligand et al., 2010). The mutation was an insertion of a single base pair between the 6th and 7th codons of *GNRH1*. The resulting frameshift disrupts the primary sequence of the GnRH preprohormone starting at the 7th amino acid, and thus the GnRH decapeptide was predicted not to be synthesized. Indeed, cells transfected with the mutant *GNRH1* allele failed to produce measurable amounts of GnRH.

The proband had micropallus, absence of pubertal development at 18 years, hypogonadotropic hypogonadism, and an intact sense of smell. His sister, who was also homozygous for the frameshift mutation in *GNRH1*, also had normosmic hypogonadotropic hypogonadism and had a normal response to exogenous pulsatile GnRH administration. The family originated from a village in Romania, and heterozygous family members had normal pubertal development.

Our group, after screening over 300 probands with normosmic GnRH deficiency for mutations in *GNRH1*, identified one proband with a homozygous frameshift mutation (Chan et al., 2009). The mutation was the deletion of a single base pair in the codon for the 6th amino acid of the GnRH decapeptide; this frameshift mutation disrupts the C-terminus of GnRH, a region essential for its function. The proband had a history of microphallus and cryptorchidism, absence of pubertal development at 13.5 years, and had an intact sense of smell. His parents, who came from the same village in Armenia, reported normal pubertal development.

3.1.1 Biological lessons from patients with homozygous *GNRH1* mutations—

The finding of homozygous frameshift mutations in two probands with isolated GnRH Deficiency established null mutations in *GNRH1* as a cause of this condition in the human. The simple fact that these patients were born healthy at term counters earlier speculation that GnRH may be essential for placental function and fetal survival. Similarly, the absence of significant non-reproductive phenotypes in these patients argues against a major function for GnRH in other organ systems.

Bouligand et al. (2009) emphasized the fact that the patients with homozygous *GNRH1* mutations had normal prolactin levels, thus addressing a long-standing controversy about a putative function of the GnRH-associated peptide (GAP). Nikolics et al. reported in 1985 that GAP is an inhibitor of prolactin release and that administration of neutralizing antibodies against GAP to rabbits causes elevation of serum prolactin (Nikolics et al., 1985). Some groups have reported similar results (Chavali et al., 1997; Milton et al., 1992), while other groups have been unable to replicate these findings (Chandrashekar et al., 1988; Ishibashi et al., 1987; Pohl et al., 1988; Thomas et al., 1988; Yu et al., 1989). These findings prompted Moretuzzo et al. (1992) to screen for *GNRH1* deletions by Southern blotting in patients with hyperprolactinemia; they identified no gross changes in 15 patients. In *hpg* mice, serum prolactin is not different from that of wild-type mice (Charlton et al., 1983). Similarly, prolactin levels were normal in the patients with homozygous *GNRH1* mutations (Bouligand et al., 2009; Chan et al., 2009) arguing against a major physiologic role for the *GNRH1* gene products, GnRH and GAP, in regulating human prolactin secretion.

These lessons from patients with homozygous *GNRH1* mutations are valuable, if largely confirmatory of observations already made in the *hpg* mouse. It is very possible that additional lessons will be learned as more homozygous *GNRH1* mutations are discovered (see section 6). For now, the more interesting findings arguably lie in the discovery of heterozygous variants in *GNRH1* associated with GnRH-deficient conditions.

3.2 Heterozygous *GNRH1* variants

Our group identified four heterozygous rare sequence variants in *GNRH1* in probands with isolated GnRH deficiency (Chan et al., 2009). In two of these pedigrees, the heterozygous *GNRH1* variants segregated with GnRH-deficient conditions (Figure 2).

One variant, R31C, substitutes a cysteine for an arginine in the 8th amino acid of the GnRH decapeptide; this conserved residue is essential for GnRH activity (Lu et al., 2007; Millar et al., 1989). This variant was found not only in a proband with normosmic IHH, but also in her two daughters (fraternal twins) who also had IHH as well as in a niece who carried a presumptive diagnosis of hypothalamic amenorrhea but whose diagnosis has subsequently been revised to IHH (Figure 2; H. Lavoie, personal communication). The proband also has two aunts with IHH, but unfortunately DNA samples were not available to track segregation.

This pedigree suggests that the R31C variant acts in a dominant fashion to cause IHH, which could occur through a number of theoretical mechanisms. Haploinsufficiency is one formal

possibility, but this is ruled out by the absence of any reproductive phenotype in heterozygous carriers of the two *GNRH1* frameshift mutations. A second possibility is that R31C is not merely a loss-of-function mutation but rather a dominant mutation. Precedent for this possibility comes from autosomal dominant neurohypophyseal diabetes insipidus, caused by mutations in *AVP*, the gene that gives rise to vasopressin. The products of these mutant genes are neurotoxic and cause degeneration of vasopressin neurons and the development of diabetes insipidus (Christensen 2007). Similarly, mutations in the *INS* gene, which gives rise to insulin, can cause autosomal dominant diabetes mellitus (Støy et al., 2007). Many of these *INS* mutations result in the addition or loss of cysteine residues, leading to abnormal disulfide bonding, endoplasmic reticulum stress, and cell death (Colombo et al., 2008). Mutations in *GNRH1* could similarly act in a dominant fashion to cause IHH, particularly the R31C mutation, which creates a new cysteine residue. A third possibility is that a heterozygous mutation in *GNRH1* could act in combination with other genetic and/or environmental factors to cause a reproductive phenotype. This possibility seems likely for a second variant, V18M for several reasons.

The V18M variant lies within the signal peptide of the GnRH preprohormone. The family harboring this change had previously been found to have a frameshift mutation in the X-linked gene *NROB1* (previously called *DAX1*; Figure 2; Habiby et al., 1996). Hemizygous mutations of *NROB1* in male patients cause hypogonadotropic hypogonadism and adrenal gland hypoplasia but heterozygous female carriers of *NROB1* mutations have been reported to be asymptomatic. This particular pedigree was notable for the finding that three female carriers of *NROB1* had delayed puberty (Figure 2). The *GNRH1* V18M mutation was found in all three of these female carriers. This finding raised the intriguing possibility that the normally silent heterozygous mutation in *NROB1* acted in combination with the heterozygous *GNRH1* variant to cause delayed puberty.

Whether these heterozygous mutations in *GNRH1* and *NROB1* truly acted together to cause delayed puberty in this pedigree may be difficult to prove with certainty. Nevertheless, this possibility illustrates two important concepts: an oligogenic basis for GnRH-deficient conditions and an expanded phenotypic spectrum for genes originally implicated in isolated GnRH deficiency.

4. Isolated GnRH deficiency as an oligogenic disorder

As detailed in Chapter XX, the genes that cause GnRH deficiency can be broadly divided into two groups: those that affect early GnRH neuronal development and migration, i.e. neurodevelopmental genes, and those that affect later GnRH neuronal function, i.e. genes that function in a more classical neuroendocrine pathway. Because of the close association between the olfactory system and the development and migration of GnRH neurons, genes involved in these processes are generally associated with Kallmann syndrome (KS), the combination of anosmia and IHH. In contrast, genes that influence later GnRH neuronal function are not thought to have a role in olfactory development and thus are typically associated with normosmic forms of IHH. *GNRH1* falls in this latter group with evidence coming again from the *hpg* mouse, which has normal number and location of GnRH neurons (Gill et al., 2008). Furthermore, gross olfactory defects have not been identified in *hpg* mice (McQueen et al., 1988). Thus, our group's initial strategy for identifying mutations in *GNRH1* was to screen only patients with normosmic IHH.

An underlying assumption of this broad classification is that the genetic basis for an individual's IHH lies in a single gene, i.e., that GnRH deficiency is a monogenic disorder. However, there is growing evidence that mutations in two or more genes can work in combination to produce GnRH-deficient conditions. For instance, our group has

demonstrated that, of patients with an identifiable coding sequence mutation in one of 8 genes responsible for isolated GnRH deficiency, 11% carry mutations in at least one other of these genes as well (Sykietis et al., 2010). This 11% prevalence of oligogenicity in GnRH deficiency will almost certainly rise as more GnRH deficiency genes are discovered and analyzed. Thus, *GNRH1* mutations could work in combination with other IHH genes to cause GnRH-deficient conditions. Because these other IHH genes could affect olfaction, this possibility challenges our initial assumption that *GNRH1* mutations would be found only in normosmic individuals.

We have therefore expanded our screening for mutations in *GNRH1* to patients with KS. To date, we have screened 229 patients with KS and have found no rare sequence variants in *GNRH1*. However, just as large numbers of normosmic IHH patients were required before clear *GNRH1* mutations were found, it may be that ongoing screening of KS patients will reveal mutations in *GNRH1*.

5. Expanding the phenotypic spectrum for GnRH-deficiency genes

Several groups have hypothesized that mutations in genes that cause isolated GnRH deficiency may also cause less severe GnRH-deficient conditions, such as constitutional delay of growth and puberty and hypothalamic amenorrhea.

Individuals with constitutional delay of growth and puberty have a significantly delayed onset of puberty. However, once started, puberty proceeds normally and reproductive function is maintained in adulthood. Sedlmeyer et al. (2005) sequenced *GNRH1* in 48 patients with constitutional delay and identified 4 heterozygous variants: 2 common polymorphisms and 2 rare variants that, though not formally tested, were unlikely to affect gene function (a synonymous change and a change in the 5' untranslated region of *GNRH1*). Our group has screened 40 probands with constitutional delay and has identified no rare sequence variants in *GNRH1* (Table 1).

Hypothalamic amenorrhea (HA) is an acquired condition in which stress, excessive exercise, and/or inadequate caloric consumption suppresses GnRH release. Our group has recently shown that 7 of 55 patients with HA carried heterozygous mutations in four genes already implicated in isolated GnRH deficiency: *FGFR1*, *PROKR2*, *GNRHR*, and *KALI* (Caronia et al., 2011). While *GNRH1* was not initially screened in this cohort, our group has subsequently sequenced *GNRH1* in 24 patients with HA and found no rare sequence variants (Table 1). Thus, mutations in *GNRH1* do not appear to be a common cause of either constitutional delay or hypothalamic amenorrhea.

6. The rarity of *GNRH1* mutations and lessons for human genetics

Homozygous mutations in *GNRH1* are a rare cause of normosmic IHH. We screened an additional 92 probands with normosmic IHH and have not identified any additional homozygous or heterozygous variants. The presence of 2 homozygous *GNRH1* mutations in ~ 600 pedigrees collectively screened by various groups (Table 1) gives a prevalence of mutations in the GnRH gene in normosmic GnRH deficiency of 0.33% (95% CI <0.01-1.3%). Furthermore, because both cases of homozygous *GNRH1* mutations came from special, endogamous populations, the prevalence of *GNRH1* mutations in more general populations is likely to be even lower. Why are *GNRH1* mutations so rare, particularly when mutations in *GNRHR*, which encodes the GnRH receptor, are among the most common mutations found in IHH (see Chapter XX)?

Any answer to this question is speculative, but there are several theoretical possibilities. The hypothesis that homozygous *GNRH1* mutations are not compatible with life was essentially

disproved by the finding of live *GNRH1* homozygotes, but it is possible that these individuals carry protective factors that allowed them to survive what would otherwise be a lethal mutation.

A more likely possibility is that mutations in *GNRH1* are subject to negative selection and rapidly eliminated from populations. Clearly, this would be true for homozygous *GNRH1* mutations, as treatments to allow individuals with GnRH deficiency to achieve fertility have become available only in the past several decades (see Chapter XX). Our finding of heterozygous *GNRH1* mutations segregating with reproductive phenotypes suggests that even heterozygous mutations may confer a reproductive disadvantage, creating further selective pressure to eliminate *GNRH1* mutations from the population.

The candidate-gene approach that was used to find *GNRH1* mutations will soon be made obsolete by the advent of affordable whole-exome and whole-genome sequencing. These techniques will inevitably lead to the discovery of more mutations in *GNRH1* and may produce answers for several of the questions raised in this review. Identifying *GNRH1* mutations in patients with other, more common reproductive disorders may reveal new pathophysiological mechanisms by which *GNRH1* mutations can disrupt GnRH biosynthesis and GnRH neuronal function, expand the spectrum of diseases caused by *GNRH1* mutations, and further demonstrate the role of oligogenicity in isolated GnRH deficiency. It may be even more telling to determine the frequency of *GNRH1* mutations in healthy controls and in other disease cohorts. A low frequency of *GNRH1* variants in healthy controls would support the hypothesis that *GNRH1* variants are subject to negative selection. An unexpected association of *GNRH1* mutations with a non-reproductive disorder would be particularly exciting, as it would reveal hitherto unappreciated functions of GnRH or the GnRH-associated peptide. Thus, while much is known about the role of *GNRH1* in the neuroendocrine control of reproduction, more surprises about *GNRH1* may be yet to come.

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Abbreviations

IHH	idiopathic hypogonadotropic hypogonadism
KS	Kallmann syndrome
RSV	rare sequence variant

- Homozygous mutations in *GNRH1* are a rare cause of human isolated GnRH deficiency.
- Heterozygous mutations in *GNRH1* may also contribute to isolated GnRH deficiency.
- Some heterozygous *GNRH1* mutations may have dominant effects.
- *GNRH1* mutations may also interact with other gene mutations to produce phenotypes.
- These findings illustrate GnRH's essential and non-redundant role in reproduction.

Figure 1.

The accelerating pace of discovery in GnRH biology. Years in which critical discoveries were made and in which genes were found to be cause GnRH deficiency are indicated.

Figure 2.

Pedigrees with heterozygous *GNRH1* variants. A. Pedigree of a family with the R31C variant. B. Pedigree of a family with the V18M variant in *GNRH1* and a frameshift mutation in *NR0B1/DAX1*. Reprinted with modification from Chan et al., 2009.

Table 1
Screening for mutations in *GNRH1* in patients with GnRH-deficient conditions

Year	Probands/Pedigrees	Method (s)	Findings	Reference
1989	7 nIHH 6 KS	Southern blotting	- -	Weiss et al., 1989
1990	1 KS	Southern blotting, sequencing	W16S [†]	Nakayama et al., 1990
1991	2 nIHH 2 KS	sequencing	- W16S [†]	Weiss et al., 1991
1992	1 KS 11 unspecified HH	Southern blotting	- -	Layman et al., 1992
2003	4 nIHH	sequencing	-	Bo-Abbas et al., 2003
2005	48 CD	sequencing	c.1-545T>C* W16S [†] Q59Q* E47D [†]	Sedlmeyer et al., 2005
2005	26 nIHH	sequencing	W16S [†] (x4), W16S [†]	Vagenakis et al., 2005
2006	26 nIHH	sequencing	-	Topaloglu et al., 2006
2008	28 nIHH 44 KS 28 unspecified HH	MLPA	- - -	Pedersen-White et al., 2008
2009	~ 150 nIHH	sequencing	p.L7TfsX36*	Bouligand et al., 2009, Bouligand et al., 2010
2009	310 nIHH	sequencing	G29GfsX12* R31C* R73X* T58S* V18M*	Chan et al., 2009
2010	55 nIHH 80 KS	MLPA	- -	Trarbach et al., 2010
2011	92 nIHH 229 KS 40 CDGP 24 HA	sequencing	- - - -	unpublished data

nIHH, normosmic idiopathic hypogonadotropic hypogonadism; KS, Kallmann syndrome; HH, hypogonadotropic hypogonadism; CDGP, constitutional delay of growth and puberty; HA, hypothalamic amenorrhea; MLPA, multiplex ligation-dependent probe amplification. Bold lettering, homozygous changes. Asterisk, changes not reported in dbSNP.

[†]dbSNP build 133 reports a heterozygote frequency of 38.7% for W16S and 2.5% for E47D (and 2.2% for F61F and 50% for R73Q, two other common polymorphisms in the *GNRH1* coding region).