The Role of the Prokineticin 2 Pathway in Human Reproduction: Evidence from the Study of Human and Murine Gene Mutations

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A widely dispersed network of hypothalamic GnRH neurons controls the reproductive axis in mammals. Genetic investigation of the human disease model of isolated GnRH deficiency has revealed several key genes crucial for GnRH neuronal ontogeny and GnRH secretion. Among these genes, prokineticin 2 (*PROK2*), and PROK2 receptor (*PROKR2*) have recently emerged as critical regulators of reproduction in both mice and humans. Both *prok2*- and *prokr2*-deficient mice recapitulate the human Kallmann syndrome phenotype. Additionally, *PROK2* and *PROKR2* mutations are seen in humans with Kallmann syndrome, thus implicating this pathway in GnRH neuronal migration. However, *PROK2*/*PROKR2* mutations are also seen in normosmic GnRH deficiency, suggesting a role for the prokineticin signaling system in GnRH biology that is beyond neuronal migration. This observation is particularly surprising because mature GnRH neurons do not express PROKR2. Moreover, mutations in both *PROK2* and *PROKR2* are predominantly detected in the heterozygous state with incomplete penetrance or variable expressivity frequently seen within and across pedigrees. In some of these pedigrees, a "second hit" or oligogenicity has been documented. Besides reproduction, a pleiotropic physiological role for PROK2 is now recognized, including regulation of pain perception, circadian rhythms, hematopoiesis, and immune response. Therefore, further detailed clinical studies of patients with *PROK2*/*PROKR2* mutations will help to map the broader biological role of the PROK2/PROKR2 pathway and identify other interacting genes/proteins that mediate its molecular effects in humans. **(*Endocrine Reviews* 32: 225–246, 2011)**

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Abbreviations: Bv8, *Bombina variegata* 8; GnRHR, GnRH receptor; GPCR, G protein-coupled receptor; KS, Kallmann syndrome; MIT-1, mamba intestinal toxin; NFAT, nuclear factor of activated T cells; nIHH, normosmic idiopathic hypogonadotropic hypogonadism; PKC, protein kinase C; PROK2, prokineticin 2; PROKR2, prokineticin receptor 2; SCN, suprachiasmatic nucleus.

I. Introduction

In mammalian species studied to date, a sparsely populated yet widely dispersed network of approximately 1500 GnRH neurons residing in the hypothalamus synchronize and coordinate the synthesis of GnRH and thus serve as the body's "pilot light of reproduction" (1). Studies in rodents show that the majority of GnRH neurons reside in the medial preoptic area of the hypothalamus and project their axonal processes into the median eminence of the hypothalamus (2). Coordinated by unknown mechanisms across this neural network, GnRH is secreted in a pulsatile fashion into the hypophyseal portal circulation (3). GnRH neurons are thus strategically positioned in the hypothalamus (*e.g.*, medial preoptic area) to facilitate the receipt of inputs from both external and internal cues. The GnRH neuronal network then integrates this information to maximize the organism's reproductive efficiency by economizing efforts and delaying sexual maturation and fertility until optimal environmental and metabolic conditions exist for bearing and nourishing offspring.

Several human studies have clearly demonstrated that pulsatile GnRH secretion is fully active *in utero*, continues well into the neonatal period and early infancy, is silenced during childhood, is reactivated at adolescence signaling puberty, and then is maintained during adult reproductive life (4–9). Upon secretion, GnRH binds to its receptor [GnRH receptor (GnRHR)] on the anterior pituitary gonadotropes and stimulates both the synthesis and secretion of the two pituitary dimeric glycoprotein hormones, LH and FSH. These gonadotropic hormones then functionally bifurcate the gonads into their steroidogenic components (Leydig cells in males, and thecal cells in females) that bear LH receptors and the gametogenic supporting cells (Sertoli cells in males, and granulosa cells in females) that bear FSH receptors. Collaboratively, these gonadotropins and their respective gonadal compartments govern the appearance of secondary sexual characteristics and ultimately maturation of the germ cells in both sexes.

Defective development of the GnRH neuronal network or impaired biosynthesis, secretion, and/or action of GnRH results in the clinical syndrome of isolated GnRH deficiency. Clinically, this condition can present either as Kallmann syndrome (KS) when it is associated with anosmia, thus signaling a developmental defect in olfactory bulb neurogenesis and GnRH neuronal migration or as normosmic idiopathic hypogonadotropic hypogonadism (nIHH) when olfaction is normal, indicating a functional defect at the level of the hypothalamo-pituitary axis. In the last decade, considerable progress has been made by leveraging genetic approaches in human GnRH deficiency that have permitted a dissection of the genetic cascade underlying GnRH ontogeny in humans (10). Significant insights into the embryological fate specification, migration, secretion, and action of GnRH neurons have resulted from these discoveries. Recently, a rich vein of mutations in a novel ligand-receptor family, prokineticin 2 (*PROK2*) and its receptor, prokineticin receptor 2 (*PROKR2*), have been described in subjects with GnRH deficiency (11–18). This review will provide an overview of the known genetic causes of isolated GnRH deficiency and describe the emerging role played by the prokineticin 2 pathway in the neuroendocrine control of reproduction.

II. Genetic Causes of Isolated GnRH Deficiency in Humans

A. Historical overview of isolated GnRH deficiency

In 1849, Aureliano Maestre de San Juan, a Spanish pathologist, first documented the association of hypogonadism and absence of the olfactory system during the autopsy of a 40-yr-old man (19). Subsequently, postmortem documentation of the association of hypogonadism with defective olfactory system was also reported by Weidenreich in 1914 (20) and Altmann in 1930 (21). Almost 100 yr from its first documentation, Franz J. Kallmann identified the symptoms of hypogonadism and anosmia as a clinical entity and noted its familial nature implying a genetic etiology (22). Since then, GnRH deficiency with the presence of anosmia has been designated as Kallmann syndrome (KS).

In the following decade, De Morsier expanded these observations and confirmed the inherited nature of this disorder that he termed "olfactogenital dysplasia" (23). Multiple lines of investigations in the early 20th century laid the foundations for understanding the role of the hypothalamus and pituitary in regulation of gonadal function in humans. With urinary gonadotropin assays initially (24) and later with the availability of sensitive RIAs to measure LH and FSH (25–27), it was recognized that some patients with hypogonadism had inappropriately low levels of gonadotropins despite their hypogonadal state. Because the level of all other anterior pituitary hormones was normal, the condition was initially referred to as isolated gonadotropin deficiency (28, 29). The seminal discovery of GnRH in 1971 (30, 31) permitted the observation of variable pituitary gonadotropin responses to single boluses of ovine (32) or synthetic GnRH (28, 29) in some patients with isolated gonadotropin deficiency and suggested a hypothalamic defect in these patients. Eventually, the hypothesis of a hypothalamic defect was confirmed when a physiological regimen of exogenous pulsatile GnRH was shown to be both necessary and sufficient to trigger puberty in these patients (29, 32–34).

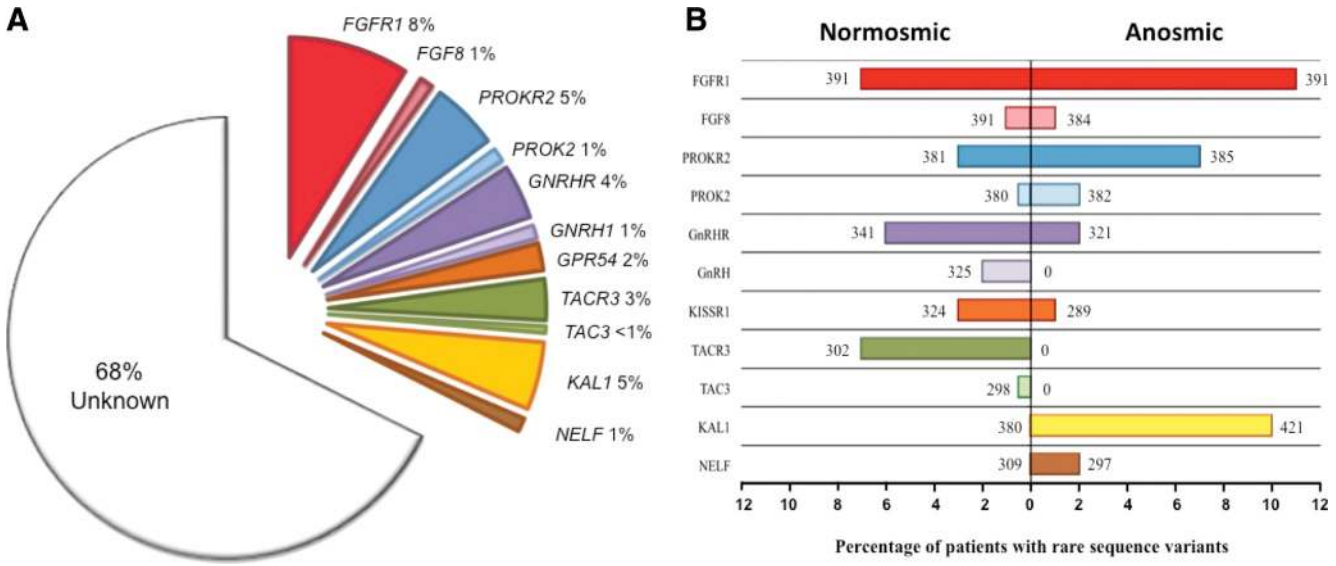

FIG. 1. Genetic causes of isolated GnRH deficiency in a large series of patients at the Massachusetts General Hospital. A, Percentage of patients (n = 397) with isolated GnRH deficiency that harbor rare sequence variants of known genes. B, Histogram of percentage of patients with isolated GnRH deficiency harboring rare sequence variants in each known gene displayed according to their olfactory phenotype. Percentage of patients with normal sense of smell is shown on the *left* of y-axis, and percentage of patients with anosmia is shown on the *right* of y-axis. The total number of patients who have been screened for each gene is given on *either side of the bar* corresponding to their olfactory phenotype. Note that the total cohort of patients shown in panel B is larger than in panel A for some genes (*i.e.*, *FGFR1*, *FGF8*, *PROK2*, and *PROKR2*). *FGFR1*, Fibroblast growth factor receptor; *FGF8*, fibroblast growth factor 8; *GPR54*, G-protein couple receptor 54; *TAC3*, tachykinin 3; *TACR3*, tachykinin receptor 3; *KAL1* Kallmann syndrome 1; *NELF*, nasal embryonic LHRH factor.

B. Genetic causes of isolated GnRH deficiency

The familial nature of GnRH deficiency was evident from the earliest studies (22, 35–39). Three possible modes of transmission for isolated GnRH deficiency were soon recognized (OMIM 308700, 147950, and 146110): autosomal recessive, autosomal dominant, and X-linked transmission. The study of an Italian pedigree in which five of six males were affected by ichthyosis and KS offered the first genetic clue for the etiology of GnRH deficiency. All affected subjects showed decreased steroid sulfatase activity, an enzyme coded by a gene located in the distal short arm of chromosome X (40, 41). Subsequently, Bick *et al.* (42) identified a terminal deletion of the X chromosome with a breakpoint at Xp22.3 in a male infant with KS, ichthyosis, X-linked recessive chondrodysplasia punctata, and choanal atresia, a deletion the infant inherited from his unaffected mother. The child subsequently died, and an autopsy revealed complete absence of the olfactory bulbs and associated tracts, choanal atresia, and a horseshoe kidney. A second male child was then conceived by the same mother. An amniocentesis showed an identical defective X-chromosome, and the pregnancy was terminated at 19 wk gestation. Postmortem examination of the fetus revealed disruption of the olfactory system associated with arrested migration of GnRH neurons into the brain at the level of the cribriform plate (43). Importantly, previous murine studies had already demonstrated that GnRH neurons originate outside the central nervous system in the medial olfactory placode during embryological development and then subsequently migrate to the hypothalamus using the olfactory system to guide their migration (44, 45). Thus, these two brothers with KS initiated the genetic era of unraveling the link between olfaction and reproduction. Two independent groups subsequently identified the first KS gene, *KAL1*, by positional cloning of the distal portion of the X-chromosome (Xp22.3) (46, 47).

After this landmark discovery of *KAL1*, several autosomal genes have now been linked to isolated GnRH deficiency. To date, roughly 32% of a large cohort of GnRH-deficient patients (n = 397) at the Massachusetts General Hospital have been linked to at least one gene mutation known to cause human GnRH deficiency (Fig. 1, A and B). This cohort covers a broad clinical spectrum of reproductive phenotypes including: 1) a mild defect of GnRH secretion altering only timing of puberty (delayed puberty); 2) an intermediary or developmental defect presenting as spontaneous puberty with subsequent development of permanent hypogonadism (acquired hypogonadotropic hypogonadism); and 3) a severe defect with complete/partial absence of puberty (48–50). Likewise, GnRH-deficient patients also display a broad spectrum of nonreproductive phenotypes including facial midline defects, renal agenesis, and skeletal abnormalities that can provide key clues as to the underlying causal gene (Table 1). Early developmental genes such as *KAL1*, *FGF8*, *FGFR1*, *NELF*, *CHD7*, *PROK2*, and *PROKR2* play a critical role in embryonic neuronal development,

TABLE 1. Genetics of isolated GnRH deficiency

Gene (chromosomal locus)	Ref.	OMIM no.	Reproductive phenotypes	Mode of inheritance	Associated phenotypes
KAL1 (Xp22.3)	47	308700	KS	X-linked	Unilateral renal agenesis, bimanual synkinesia, high arched palate
KISS1R (19p13.3)	151	604161	nIHH	Autosomal recessive	None
FGFR1 (8p11.2-p11.1)	152	136350	KS	Autosomal dominant	Cleft lip/cleft palate, skeletal anomalies (hand and foot), external ear hypoplasia, dental agenesis
FGF8 (10q.24)	153	600483	nIHH	Autosomal dominant	Cleft lip/cleft palate, skeletal anomalies (hand and foot), external ear hypoplasia, dental agenesis
PROK2 (3p21.1)	11, 12	607002	KS	?Autosomal recessive	? Circadian/sleep dysregulation, ? glucose intolerance
PROKR2 (20p.13)	11	607123	nIHH	?Autosomal recessive	? Circadian/sleep dysregulation, ? glucose intolerance
GnRH-1 (8p21-p11.2)	154	152760	nIHH	Autosomal recessive	None reported
GnRHR (4q21.2)	155	138850	nIHH	Autosomal recessive	None reported
TAC3 (12q13-q21)	156	162332	nIHH	Autosomal recessive	Microphallus, cryptorchidism, reversal of GnRH deficiency
TACR3 (4q25)	156	162330	nIHH	Autosomal recessive	Microphallus, cryptorchidism, reversal of GnRH deficiency
NELF (9q34.3)	127	608137	KS	Digenic	None reported

?, Indicates potential association.

and subjects with mutations in these genes present primarily with KS. In addition, these subjects often manifest other associated developmental anomalies (*e.g.*, cleft lip/palate seen with *FGFR1* mutations). In contrast, subjects with mutations in genes such as *KISS1R* (the receptor for the hypothalamic neuropeptide kisspeptin, which plays a critical role as gatekeeper of puberty), *GnRH1*, *GnRHR*, *TAC3*, and *TACR3* present primarily with nIHH. Interestingly, some adults with nIHH also harbor mutations in the early developmental genes (*FGF8*, *FGFR1*, *PROK2*, and *PROKR2*), suggesting an additional role for these genes in regulation of GnRH function in adulthood (Fig. 1, A and B). In addition, mutations in some genes (*e.g.*, *LEP*, *LEPR*) also result in isolated GnRH deficiency as part of more complex syndromic presentations. All of these genes are listed in Tables 1 and 2 and are reviewed extensively in other publications (51–56). This review will specifically focus on the role of the newly identified genes, *PROK2* and *PROKR2*, in the neuroendocrine control of reproduction.

III. Discovery of Prokineticin Family

The first report of prokineticin-like peptides dates back to 1980 when venom protein A was isolated from the nontoxic constituents in the venom of black mamba snake (*Dendroaspis polylepis*) (57). This peptide was subsequently renamed as mamba intestinal toxin, MIT-1 (58, 59). Similarly, an ortholog of human PROK2 was isolated

TABLE 2. Genetics of isolated GnRH deficiency associated with complex syndromes/diseases

Gene (chromosomal locus)	Ref.	OMIM no.	Reproductive phenotypes	Mode of inheritance	Associated syndrome/ phenotype
LEP (7q31.3)	157	164160	nIHH	Autosomal recessive	Severe obesity
LEPR (1p31)	158	601007	nIHH	Autosomal recessive	Severe obesity
NROB1 (Xp.21.3-p21.2)	159	300473	nIHH	X-linked	X-linked adrenal hypoplasia congenita
CHD7 (8q12.1)	160	608892	KS, nIHH	Autosomal dominant	Part of CHARGE syndrome

CHARGE syndrome, Coloboma, heart defect, choanal atresia, growth retardation, genital and ear abnormalities.

from the skin secretion of the fire-bellied toad (*Bombina variegata*). This molecule was named *Bombina variegata 8* (Bv8) to indicate its species of origin and its molecular mass of 8 kDa (60). In 2001, Zhou and collaborators (61) identified two human cDNAs that encode the human orthologs for the snake toxin MIT-1 and the toad skin-secreted protein, Bv8. The human counterparts were named *PROK1* and *PROK2* to highlight their potent and specific motility enhancement of the gastrointestinal tract (59, 61). In an attempt to enhance the clarity and maintain consistency with the nomenclature, we will use the designations of PROK1 and PROK2 for the rest of this review. Apart from its gastrointestinal effects, PROK1 was also identified as an angiogenic factor with specific effects on endocrine organs, thus earning its initial name, endocrine gland vascular endothelial growth factor (62). This name reflects the functional resemblance with vascular endothelial growth factor, a widely known angiogenic factor, although these molecules are structurally unrelated (62, 63).

Almost simultaneously with the discovery of the prokineticins, three independent research groups identified and characterized their cognate receptors (64–66). Mature *PROK1* and *PROK2* are ligands for the highly homologous (85%) G protein-coupled receptors *PROKR1* and *PROKR2*, formerly known as GPR73a and GPR73b, respectively. Intensive research of the prokineticin system over the past decade has revealed a dazzling array of physiological functions of this pathway, including regulation of circadian rhythms, metabolism, angiogenesis, neurogenesis, pain perception, muscle contractility, hematopoiesis, immune response, and reproduction (67–69). In addition, the disruption of prokineticin system has been implicated in several pathological conditions, including cancer (70, 71), immunological response (71, 72), mood disorder (anxiety/depression) (73), and cardiomyopathy (74). In the following sections, we will briefly describe the prokineticin family (PROK1/PROK2 and PROKR1/PROKR2) and then specifically focus on the role of prokineticin 2 pathway on the neuroendocrine control of reproduction.

IV. Prokineticin Ligands: PROK1 and PROK2

In contrast to the high homology exhibited by the prokineticin receptors, the ligands, *PROK1* and *PROK2*, share only 44% amino acid identity. Most of this homology resides in the N-terminal signal peptide and the distinct AVITGA sequence motifs (Fig. 2, A and B) that are highly

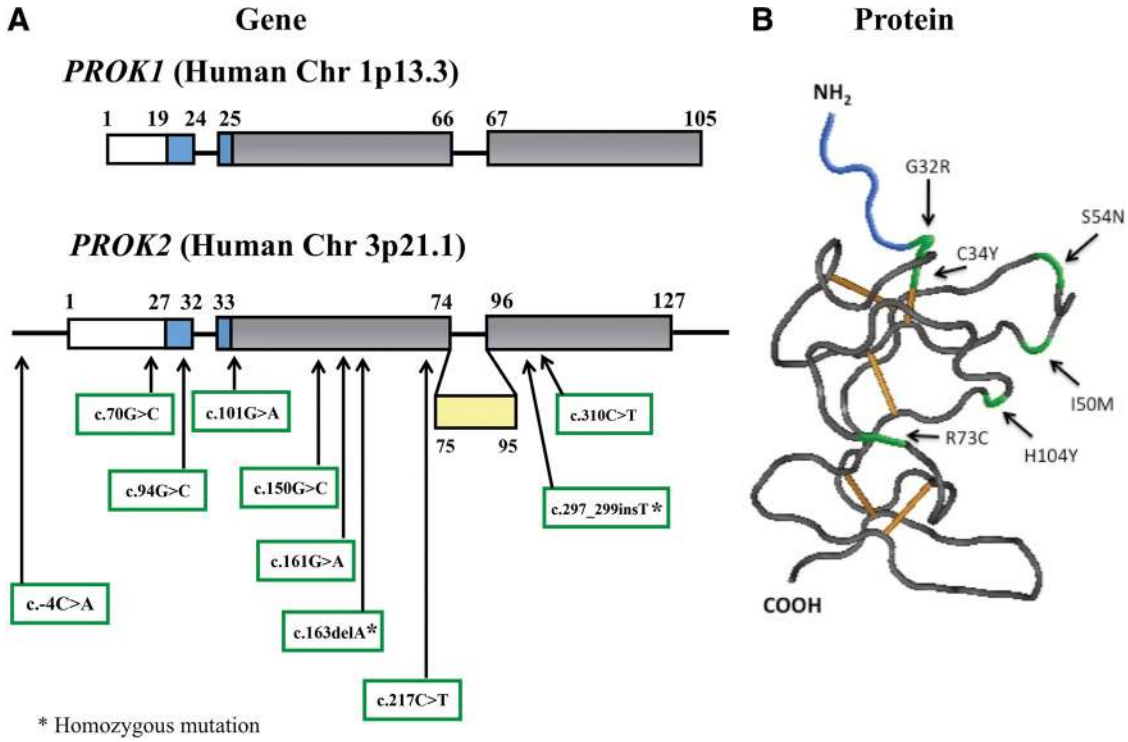

FIG. 2. Schematic representation of the gene and protein structures of the prokineticin ligands. A, *PROK1* and *PROK2* are encoded by three and four exons, respectively. Exons 1, 2, and 4 of *PROK2* gene encode a mature protein of 81 amino acids. Exon 3 of *PROK2* is represented in *yellow* and undergoes alternative splice processing. Both *PROK1* and *PROK2* encode a signal peptide that is shown in *white*. The gene sequence that encodes the AVITGA motif is shown in *blue*. The amino acid substitutions resulting in various mutations in the *PROK2* gene identified to date in GnRH deficiency patients (12, 13, 16, 18) are shown *below the schematic* of the *PROK2* gene. B, The PROK2 protein three-dimensional structure (Protein Data Bank code: 1IMT) was modeled using Cn3D 4.1 software (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). The mature prokineticin ligands exhibit a globular shape as a result of five disulfide bonds (shown in *orange*). Selected nonsynonymous mutations in *PROK2* gene identified in GnRH-deficient patients are shown in *green*. The AVITGA sequence is shown in *blue*.

preserved across several species (fish, frog, snake, and various mammalian species) (75). In view of the distinctively conserved N-terminal AVITGA sequence, Kaser *et al.* (75) proposed the term "AVIT family" to classify the prokineticins and their nonmammalian orthologs. Despite these structural similarities, *PROK1* and *PROK2* have diverse physiological effects in view of their differential anatomical distribution. Furthermore, the nonselectivity of the ligands to recognize their receptors (PROKR1 and PROKR2) results in homologous and heterologous ligand/receptor pairings that could potentially trigger divergent downstream signaling pathways (74, 76). These features suggest that the complex intracellular context results in a differential ligand/receptor coupling and, as a consequence, diverse biological actions.

A. Gene sequence

The PROK1 gene maps to regions of human chromosome 1p13.1 and mouse chromosome 3 (77). The gene is organized in three exons encoding a precursor protein of 105 amino acids and a mature form of 86 amino acids (63, 78) (Fig. 2A). The first exon encodes 19 residues corresponding to the signal peptide, and the first five amino acids of the mature protein correspond to the conserved and essential AVITGA sequence domain. The second and third exons encode for a total of ten cysteine residues, suggesting a high degree of tertiary structure to the molecule (six and four cysteine residues, respectively) (Fig. 2, A and B). Despite the highly conserved gene organization, the promoter region in human and mouse diverge, suggesting selective and unique expression patterns and perhaps diverse functions over evolution (77).

The PROK2 gene is located on human chromosome 3p21.1 and mouse chromosome 6 and is arranged in four exons (79, 80). The human and mouse region have five blocks of sequence that are highly conserved (80–100% identity), suggesting a related transcriptional regulation in these species (63, 80). The gene organization of PROK1 and PROK2 is similar except for the third exon in PROK2, which is absent in the PROK1 genomic sequence (60, 61, 80, 81) (Fig. 2A). This third exon can be inserted by alternative splicing, thereby resulting in two mature proteins (76, 80, 81) (Fig. 2A). After the signal peptide (27 amino acids) processing, *PROK2* encodes an 81-amino acid protein and a longer isoform (PROK2L) of 102 amino acids with a highly basic insert after residue 74 (80, 81) (Fig. 2A). This insert in PROK2L is rich in arginine and lysine residues and contains several potential cleavage sites for furin and other prohormone convertases (81, 82). To date, the structure and biological action of this long variant remains unknown. The PROK2 promoter region has several E-box sites that are recognized by members of the basic-helix-loop-helix family such as CLOCK and BMAL1 (83) [implicated in the role of PROK2 in circadian regulation in the suprachiasmatic nucleus (SCN)] and NGN1 and MASH1 (implicated in the role of PROK2 in the olfactory bulb) (84).

B. Protein structure

The distinctive N-terminal sequence of both PROK1 and PROK2 (the AVITGA sequence) has been implicated in receptor recognition (76). The high degree of disulfide cross-linking gives rise to a remarkably stable compact protein that is highly resistant to protease degradation (76, 85). Both proteins fold into a polarized ellipsoid structure with one side containing a net positive charge and the opposite with hydrophobic residues (85) (Protein Data Bank, accession number 1IMT) (Fig. 2B). The C- and N-terminal ends are exposed on the surface, whereas the more charged residues are buried inside the molecule (85). The disulfide bond pattern of prokineticins is similar to both colipase, a cofactor for intestinal lipid digestive enzyme lipase, and Dickkopf family members, which are extracellular proteins that organize embryonic head development in *Xenopus* through the regulation of Wnt/catenin signaling pathways and are important to bone development (86).

C. Anatomical localization

PROK1 and PROK2 are expressed in an impressive array of organs including brain, ovary, testis, placenta, adrenal cortex, peripheral blood cells, intestinal tract, heart, and bone marrow (69, 72) (Tables 3 and 4). Despite their similar pattern of expression, PROK1 and PROK2 have a unique temporal and spatial tissue distribution of expression (87, 88). For example, PROK1 is predominantly expressed in steroidogenic organs: ovary > testis > adrenal cortex > placenta (62); whereas PROK2 is mainly (but not exclusively) expressed in the central nervous system and nonsteroidogenic cells of the testes (87, 88).

From a human reproductive point of view, PROK1 has been reported to have regulatory effects on the gonads (68), whereas PROK2 plays a major role in olfactory bulb development and GnRH neural migration (12, 89) (see *Section VII.A*). In the human ovary, PROK1 is strongly expressed in the granulosa cells of primordial and primary follicles and then in theca cells during early to mid luteal phase (87, 90). Similar patterns of PROK1 expression have been shown in bovine ovaries (91) and also in primates such as the cynomolgus monkey (*Macaca fascicularis*) and the chimpanzee (*Pan troglodytes*) (62). In contrast, PROK2 is undetectable in human ovary (87). In addition to the ovary, PROK1 is also detected in endo-

TABLE 3. Expression pattern of PROK1, PROK2, PROKR1, and PROKR2 in tissues associated with reproductive function

Tissue	Ref.	PROK1	PROK2	PROKR1	PROKR2
Brain	82, 116		Olfactory bulb, hypothalamus (medial preoptic area, arcuate nucleus)	Olfactory bulb and ventricles, hypothalamus (arcuate nucleus, mammillary bodies)	Olfactory bulb, piriform and entorhinal cortex, band of Broca, hypothalamus (lateral preoptic area, paraventricular nucleus, arcuate nucleus, median eminence, mammillary nucleus, subfornical organ)
Pituitary	64, 65	Present		Present	Present
Ovary	64, 87	Granulosa and theca cells		Capillary endothelial cells	Capillary endothelial cells
Uterus	65, 95, 97	Glandular epithelium, stromal and smooth muscle cells	Glandular epithelium, stromal and smooth muscle cells	Glandular epithelium, stromal and smooth muscle cells	Glandular epithelium, stromal and smooth muscle cells
Placenta	93, 94	Present		Present	
Testis	63, 64, 81, 102	Leydig cells	Primary spermatocytes	Endothelial cells of interstitium	Endothelial cells of interstitium
Prostate	62, 64	Prostate cancer		Present	
Human fetal tissue	61, 66	Present	Present	Present	Present

metrial tissue, where it reaches a maximum level of expression during "the implantation window" of women in reproductive age (72, 92). In contrast, PROK2 expression in the endometrium remains constant across the menstrual cycle (92). During the first trimester of pregnancy, PROK1 levels increase further in the decidualized endometrium compared with midluteal phase of the menstrual cycle (93–95). PROK1 is also highly expressed in term placentas (syncytiotrophoblast and cytotrophoblast), fetal endothelium, and macrophages (96). However, it is rarely detected after menopause or in endometrial carcinoma patients (97). The physiological implications of PROK1 presence in the ovary, uterus, and in various tissues of pregnancy have been comprehensively studied as discussed by others (68, 93, 95). Recently, several reports indicate the involvement of PROK1 signaling in human ectopic endometriosis (98, 99), in ectopic pregnancy (100), and in the immune response of pregnancy (96, 101). PROK1 has also been proposed as a potential biomarker to predict endometrial receptivity in patients undergoing *in vitro* fertilization (95).

In males, PROK1 is abundantly expressed in the testes from embryonic wk 14 until birth during early testicular development (102). In adult men, in keeping with its steroidogenic theme, PROK1 is expressed in Leydig cells (102), whereas PROK2 expression is restricted to the primary spermatocytes in both humans and mice (81, 87). The PROK2L variant is also expressed in the testes (81). Thus, in the testes, the prokineticin ligands are considered to be angiogenic and mitogenic/survival factors and are

TABLE 4. Expression pattern of PROK1, PROK2, PROKR1, and PROKR2 in nonreproductive tissues

Tissue	Ref.	PROK1	PROK2	PROKR1	PROKR2
Brain	65, 88	Tractus solitarium, cerebellum	Basal ganglia (accumbens nucleus, islands of Calleja), hypothalamus (suprachiasmatic nucleus), medial amygdala, mesencephalon (Edinger Westphal)	Hypothalamus (zona incerta), mesencephalon	Hippocampus, globus pallidus, amygdala, thalamus, hypothalamus (SCN)
Thyroid gland	64			Present	Present
Thymus	65	Present		Present	Present
Salivary gland	64	Present			
Heart	141	Cardiovascular tissue, cardiac cells	Cardiovascular tissue, cardiac cells	Cardiovascular tissue, cardiac cells	
Lung	65	Present		Present	Present
Liver	65, 161		Kupffer cells	Kupffer cells	Kupffer cells
Spleen	64, 65	Present		Present	Present
Kidney	62, 65, 87	Epithelial tubules		Present	Endothelial cells
Adrenal gland	64, 65, 162	Glomerulosa and fasciculate cells	Glomerulosa, fasciculate, and endothelial cells	Glomerulosa, fasciculate, and endothelial cells	Glomerulosa and fasciculate cells
Pancreas	64, 78, 163	Pancreatic islets and stellate cells		Vascular endothelial cells	Vascular endothelial cells
Stomach	61, 65, 164	Present		Present	
Intestinal tract	72, 165	Enteric plexus, mucosa of embryonic gut	Enteric plexus	Enteric neural crest cells; plexus cells of Jejunum, ileum, ileocecum, and colon	Enteric plexus of ileocecum
Skeletal muscle	65, 66	Present		Present	Present
Adipocytes	65	Present		Present	Present
Bone marrow and peripheral blood	71, 166	B and T cells	Hematopoietic stem cells, monocytes, neutrophils and dendritic cells	Hematopoietic stem cells, mature blood cells	Hematopoietic stem cells, mature blood cells

potentially involved in the high rate of endothelial cell turnover (87). The expression pattern of PROK1 and PROK2 in reproductive tissues and their expression in nonreproductive tissues are tabulated in Tables 3 and 4, respectively.

V. Prokineticin Receptors: PROKR1 and PROKR2

PROKR1 and PROKR2 are closely related members of the GPCR family (61, 64, 65). The anatomic distribution of prokineticin receptors provides insights to understand the multiple physiological roles that are already attributed to the prokineticins (69, 103).

A. Gene sequence

The *PROKR1* gene is located in human chromosome 2p13.3 and mouse chromosome 6. The *PROKR2* gene is mapped to human chromosome 20p13 and mouse chromosome 2. Both receptors are encoded by two exons separated by an intron located at the border of transmembrane domain III within the common DRY sequence motif (61). The primary sequence of both prokineticin receptors is remarkably conserved, displaying approximately 85% identity in the amino acid sequence (61, 65, 66).

B. Protein structure

The prokineticin receptors belong to the rhodopsin family of GPCRs. Despite the absence of a crystal structure, the knowledge about the prokineticin signaling pathway has been inferred through pharmacological and biochemical approaches (67, 72, 75). Systems biology approaches are emerging as useful tools to understand the enormous complexities of GPCR signaling (104). Among GPCRs, crystal structures have been solved only for the bovine rhodopsin (105) and the human β-adrenergic receptors (106). The structural knowledge of other GPCR family members has been deduced primarily by homology modeling. Seven trans-membrane- spanning regions in the cell surface characterize the GPCR family. Extracellular agonist molecules (*i.e.*, peptides, neurotransmitters) induce conformational changes, allowing the interaction with heterotrimeric G proteins associated with downstream effectors (107). Most importantly, GPCR-interacting proteins exquisitely regulate the GPCR signal transduction, receptor trafficking, and stability in the cell surface. Because the prokineticin ligands and receptors are differentially expressed in distinct tissues, their intracellular effects are customized in each cell type (107) (Tables 3 and 4).

C. Anatomical localization

The anatomical localization of the prokineticin receptors has been extensively studied, and in comparison to their ligands, both PROKR1 and PROKR2 have a considerably wider tissue distribution (65, 66, 74, 108–110) (Tables 3 and 4). The prokineticin receptors can be detected as early as embryonic d 7 in the mouse (65, 67), suggesting their involvement in early development. PROKR2 is abundantly expressed in the forebrain and testes, whereas PROKR1 is mainly expressed in peripheral tissues such as spleen, prostate, pancreas, heart, and blood cells. As a rule of thumb, tissues with high levels of PROKR1 frequently exhibit low to undetectable levels of PROKR2, and vice versa. In general, prokineticins act as diffusible messengers that reach their receptors in target cells through a paracrine mechanism (*e.g.*, PROK1 is expressed by Leydig cells, whereas PROKR1 and PROKR2 are expressed in endothelial cells of interstitial spaces in the testes). Considering that prokineticin receptors are frequently expressed in the vascular endothelium of endocrine organs, it is tempting to speculate that PROKR1 and/or PROKR2 may play a critical role in hormone secretion and/or hematopoetic regulation (87, 111). An increasing number of reports indicate that prokineticin receptor activation promotes cell migration in multiple cell types including neural progenitors in zones with active neurogenesis (89) and peripheral leukocytes involved in inflammatory response and tumor growth (71, 112).

From a reproductive perspective, targeted deletions of *prokr2* in mice show that this system is crucial for olfactory bulb morphogenesis and consequently for GnRH neuronal migration (12, 113). Accordingly, PROKR2 mRNA is present in neural precursors generated in the subventricular zone and across the rostral migratory stream that travels toward the olfactory bulb (89, 114). This neuronal stream continuously populates and replenishes the olfactory bulb, one of the few areas in the central nervous system that is continually regenerating in adult life (115). Within the olfactory bulb, PROK2 is produced and secreted to attract PROKR2-expressing cells during migration (89). Apart from the olfactory bulb, PROKR2 is expressed in the hypothalamic regions that have the highest density of GnRH-expressing neurons, such as the diagonal band of Broca, preoptic area, the paraventricular nucleus, the arcuate nucleus, and the median eminence (108, 116). PROK2 is also distinctly expressed in the SCN in a circadian fashion, and the expression of PROKR2 is complementary with significant expression in prime SCN target areas in the hypothalamus, including the areas with the higher density of GnRH neurons (116). PROK2 and PROKR2 are expressed in the limbic system, although their precise role in the limbic system is unclear (108).

PROKR2 is also abundantly expressed in the medial preoptic area, the islands of Calleja, the nucleus accumbens, the amygdala, the globus pallidus, and the thalamic areas (108, 116) (Tables 3 and 4). In contrast to PROKR2, PROKR1 is moderately expressed in the olfactory system, but its physiological role in this system remains to be defined (108). PROKR1 is also detected in the arcuate nucleus, mammillary nucleus (112), astrocytes (109), and in brain blood capillary endothelial cells (110), although their functional role in these cells is yet to be determined.

Angiogenesis is a crucial function across the menstrual cycle and during pregnancy. In the ovary, in keeping with its angiogenic theme, PROKR1 and PROKR2 are mainly expressed in the capillary endothelial cells. Kisliouk *et al.* (91) demonstrated that PROKR1 (but not PROKR2) is potentially involved in macrophage activation in atretic follicles and in the regressing corpus luteum. This group had previously reported that PROKR2 expression is significantly increased under stress conditions in the endothelial cells of the corpus luteum, whereas PROKR1 mRNA levels remained unchanged (117). In the testes, both receptors are expressed in endothelial cells of the interstitial tissue, responding to PROK1 from Leydig cells and PROK2 from primary spermatocytes (87). However, PROKR1 and PROKR2 mRNA levels are not affected by sex steroid milieu (97). Interestingly, exogenous ligand delivery, either PROK1 or PROK2, produces a dramatic angiogenic effect that correlates with the high endothelial cell turnover in testes despite being a noncyclic tissue (63). In the endometrium, both receptors are abundantly expressed during the proliferative phase. However, under pathological conditions such as endometriosis, PROKR2 mRNA levels increase even more in the proliferative phase of the cycle, whereas PROK1 and PROKR1 remain unchanged (94). During pregnancy, the activation of PROKR1 by PROK1 induces the release of proinflammatory cytokines and acts as a mitogenic factor to differentiate macrophages in the human placenta during the third trimester (68, 96, 101). The anatomical expression of PROKR1 and PROKR2 in tissues associated with reproduction and nonreproductive tissues is tabulated in Tables 3 and 4, respectively.

VI. Prokineticin Signaling

Both PROK1 and PROK2 activate both receptors in the nanomolar range, although PROK2 has moderately higher affinity for both receptors (64–66). Similarly, nonmammalian prokineticin (MIT-1 and Bv8) cannot discriminate between the two receptors. However, they display considerably higher affinity with at least one order of magnitude higher compared with human prokineticins (67). The longer isoform, PROK2L, recognizes both receptors but with a 150-fold decreased affinity compared with PROK2 (76). Further studies will be needed to explore the biological actions as well as the pathophysiological implications of the various PROK2 isoforms. Most importantly, because prokineticin ligands and receptors exhibit a differential anatomical distribution (Tables 3 and 4) and selectivity to recognize their receptors (PROKR1 and PROKR2), it is possible that the intracellular context will customize downstream signaling pathways, resulting in diverse biological actions. Several recent reviews describe this topic in greater detail (67, 69, 72).

Activation of the prokineticin receptors induces intracellular calcium mobilization through several mechanisms. One of them is via Gq coupling that activates phospholipase Cβ and subsequent formation of inositol triphosphate (64) and calcium release from intracellular stores. Phospholipase C inhibition prevents the effects of PROK2 on chemotaxis of mouse macrophages, further supporting the involvement of Gq coupling of the prokineticin receptors (118). In contrast, PROK2 induced-ERK phosphorylation and chemotaxis of human monocytes are inhibited by pertussis toxin, suggesting involvement of the Gi proteins and indicating a species-specific variation of the intracellular effects. Intracellular calcium stimulation by PROK1 also activates the calcineurin pathway, which induces dephosphorylation of the transcription factor, NFAT (nuclear factor of activated T cells), followed by nuclear translocation of NFAT and regulation of gene transcription (101). It remains to be seen whether prokineticin 2 signaling may involve the calcineurin-NFAT pathway. PROK1 and PROK2 expression can be modulated by hypoxia-induced factor-1α and potentially implicated in vascular remodeling (103).

In the peripheral nervous system, PROKR1 is expressed in the dorsal root ganglion (119). In the dorsal root ganglion, prokineticin receptors increase intracellular calcium by inducing the transient receptor potential vanilloid 1 channels in a dose-dependent fashion and are followed by subsequent translocation of protein kinase C (PKC) to the neuronal membrane (119). Furthermore, cross talk between the prokineticin pathway activated PKC and the prostaglandin E2-activated cascade (adenylate cyclase/cAMP/protein kinase A) is thought to contribute to the hyperalgesic effect of PROK2, although the mechanism connecting PKC and cAMP remains elusive (67).

Because the MAPK pathway activation is critical for angiogenesis, Lin *et al.* (78) examined the prokineticin pathway-induced activation of the p44/p42 MAPK pathway in transfected cell lines. In this report, prokineticin

signaling was shown to activate MAPK pathway, suggesting involvement of prokineticin receptor coupling to Gi proteins. Likewise, in cerebellar granule cell cultures, PROK2 stimulates neuronal survival and protects the cultured neurons against excitotoxic death by activating MAPK/phosphatidylinositol-3 kinase pathways (120). In Ishikwawa endometrial epithelial cell lines, PROK1-PROKR1 signaling has been shown to induce inositol phosphate mobilization with sequential phosphorylation of c-Src, epidermal growth factor receptor-MAPK-ERK pathway (95).

Thus, precise intracellular actions of the prokineticins are likely to be determined by the differential interactions with Gq, Gi, and Gs coupling of the receptors in specific cells and at specific developmental time frames (110). However, currently there is sparse evidence of other GPCR modulators regulating prokineticin signaling. Mutations in *PROK2* and *PROKR2* in humans have been extremely useful in providing critical insights into the signaling cascade (see *Section VII*). Further study of these human mutations is likely to facilitate identification and expansion of the signal pathway, including effectors and interacting proteins, and this will be imperative to help understand the cell-specific and species-specific biological actions of the prokineticin 2.

VII. Prokineticin 2 Pathway and Neuroendocrine Control of Reproduction

Although our knowledge about the precise molecular mechanisms underlying the biological role of PROK2 in the reproductive neuroendocrine axis remains in its infancy, PROK2 is incriminated by the combination of its clear causation in genetically manipulated mice (11, 12, 113) and its association with the disease state in humans (11, 12). In addition, the role of PROK2 in neuroendocrine hormonal regulation is also supported by the localization of PROK2 in hypothalamic regions critical for GnRH action such as the preoptic area, arcuate nucleus, and median eminence (108). PROK2 is also expressed in the islands of Calleja, nucleus accumbens, amygdala, and premammillary nucleus, which are regions associated with reproductive and feeding behavior (108). In this context, Shapiro and collaborators (121, 122) propose that the above-mentioned structures participate in encoding the appropriate reward component to novel olfactory and emotional information, which is then consolidated into memories by the hippocampus. It is also clear from multiple lines of evidence that circadian signals contribute directly to the neuroendocrine control of reproduction (123, 124). PROK2 is abundantly expressed in the SCN, and PROK2-expressing neurons in the SCN extend their neural processes into the preoptic area where the GnRH neurons reside (83, 108, 116). However, this intense PROK2 expression is undetectable in the SCN during fetal life and increases after the first postnatal week, suggesting that the clock's molecular machinery requires a maturation process (125). Thus, PROK2 is proposed to modulate GnRH function in adults by acting as a key circadian output molecule from the SCN to mature GnRH neurons (116). However, direct evidence for this association in humans is still lacking. This section details the phenotypes of both mice and humans with defects in PROK2/PROKR2 genes.

A. GnRH deficiency in *PROK2* and *PROKR2* knockout mice

1. Prok2*-deficient mice*

The *prok2* knockout mice develop disrupted olfactory bulb morphogenesis and a dramatic reduction of GnRH-expressing cells in the median preoptic area, with only a few neural projections detected in the median eminence (12). These findings phenocopy the anatomical observation of *KAL1* deficiency in humans depicted by the failure of GnRH neurons to enter into the forebrain and herein forming a spherical-shape structure in the nasal septum immediately before the cribriform plate (43). Because the *KAL1* gene has never been located in the mouse genome, these mice represent the first murine model of KS. Approximately 50% of *prok2* knockout mice show asymmetric development of olfactory bulb. Although some GnRH neurons reach the hypothalamus in these mice, their numbers and/or function are insufficient to initiate reproductive axis competency. This finding suggests that GnRH neurons that reach the hypothalamus in *prok2* knockout mice may not be functional and thus implies that PROK2 may impact on GnRH neuronal integrity through additional mechanisms besides olfactory bulb neurogenesis (12, 89). Intriguingly, PROKR2 is not expressed in GnRH neurons themselves, and thus the elucidation of the molecular mechanisms underlying the PROK2/PROKR2 regulation of GnRH neuronal development/function remains a significant investigatory challenge.

The dramatic decrease of hypothalamic GnRH neurons in *prok2* knockout mice results in low levels of GnRH secretion, absent gonadotropin secretion, and impaired sexual development and fertility. Twelve-week-old *prok2* knockout males have immature testes with small seminiferous tubules that lack lumens and absent haploid spermatocytes (12). Interestingly, under normal conditions, *prok2* is heavily expressed in diploid spermatocytes (63, 81) that give rise to the haploid spermatocytes after meiotic division. Thus, it is likely that *prok2* could play a role in final stages of spermatogenesis. Female *prok2* knockout

TABLE 5. Genotypes/phenotypes of KS and nIHH subjects with *PROK2* mutations

First author (Ref.)	Patient cohort	No. affected/diagnosis	Mutations	Associated phenotypes
Dode (11)	192 KS	4/KS	Heterozygous	Severe sleep disorder, obesity
Pitteloud (12)	50 KS, 50 nIHH	1/KS	Homozygous	Type 2 diabetes, epilepsy
Cole (13)	170 KS	2/KS	Heterozygous	Synkinesia, type 2 diabetes
	154 nIHH	1/nIHH	Heterozygous	Synkinesia, type 2 diabetes
Abreu (16)	63 KS	2/KS	Both homozygous	Pectus excavatum
	44 nIHH	1/nIHH	Heterozygous	Pectus excavatum
Leroy (15)	2 Patients (case report)	2/KS	Homozygous	None
Sarfati (18)	Comparative study	3/KS	Heterozygous	Nystagmus, pectus excavatum, high arched palate, bilateral sensorineural deafness

mice exhibit disrupted estrous cycles as a consequence of incomplete follicular development characterized by the absence of mature follicles and corpora lutea (12). Interestingly, in mice and in humans with *PROK2* deficiency, ovarian function can be restored in response to gonadotropin replacement (12). Taken together, these findings indicate that *prok2* is likely to contribute to spermatogenesis in males, but its predominant role in females is in producing a hypogonadotropic state.

2. Prokr2*-deficient mice*

The reproductive phenotype of the *prokr2* knockout mice is remarkably similar to the phenotype of the *prok2* knockout mice (113). Both *prok2*- and *prokr2*-deficient mice exhibit arrest of GnRH neuronal migration in the same physiological window, and the GnRH neurons form a "fibrocellular mass" just beyond the cribiform plate immediately before their entry into the forebrain (12, 113). However, a major difference between the *prok2* and *prokr2* knockout mice is evident in the olfactory system development: all *prokr2* knockout mice have a dramatic decrease in the olfactory bulb size (113), whereas half of the *prok2*-deficient mice exhibit asymmetric olfactory bulb development (12). In sharp contrast, *prokr1* knockout mice display normal olfactory bulb development (113). *Prokr2*-deficient mice display a striking atrophy of the testes with reduced diameter of seminiferous tubules and lack of sperm in the lumen (113). In addition, histological analysis reveals the presence of spermatogonia and spermatocytes although the spermatids are absent, suggesting a potential role of PROK2 signaling in the last steps of spermatozoa maturation. In wild-type testes, *prokr2* is abundantly expressed in endothelial cells of the interstitial space in the testis, which exhibits the highest endothelial cell turnover despite being a noncyclic tissue (63, 87). This observation suggests that *prokr2* could be involved in vascular remodeling because *prokr2*-deficient mice display reduced interstitial space accompanied by small and scattered Leydig cells, compared with their wild-type littermates (113). Similarly, ovarian folliculogenesis is frequently arrested in the preantral phase, and corpora lutea are often absent in female *prok2* (12) and *prokr2* knockout mice (113). In addition, these mice also show striking atrophy of the endometrial tissue, uterus, and the epithelial layers of the vagina, which is consistent with recent reports implicating a role for *PROKR2* in the pathogenesis of ectopic endometrial human tissue (98).

B. *PROK2* and *PROKR2* mutations in isolated GnRH deficiency in humans

After the description of unexpected GnRH deficiency in murine knockouts of *prok2* and *prokr2*, human *PROK2* and *PROKR2* genes became potential candidate genes for the etiology of human GnRH deficiency. Subsequently, in a cohort of 192 unrelated KS patients, Dode *et al.* (11) reported DNA sequence changes in both *PROK2* and *PROKR2* genes. Remarkably, a significant number of these changes were in the heterozygous state (Tables 5 and 6 and Figs. 2 and 3). In this report, four patients harbored heterozygous changes in *PROK2* (one frameshift with a premature stop codon and three missense changes) (Table 5). Ten different *PROKR2* mutations (one frameshift causing premature stop codon and nine missense changes) were seen in 14 patients in heterozygous (10 subjects), homozygous (two subjects), and compound heterozygous (two subjects) states (Table 6). No functional studies of the missense mutations were reported in this study.

Subsequently, in a Portuguese family with three affected siblings with GnRH deficiency (two brothers and one sister), a homozygous deletion in *PROK2* was reported (12) (Table 5). This deletion resulted in a 27-amino acid truncated protein, which was functionally demonstrated to be biologically inactive. Interestingly, in this pedigree, both affected brothers with the homozygous mutation had KS, and the affected sister who also harbored the homozygous mutation had nIHH whereas another unaffected brother was heterozygous for the mutation, suggesting that homozygous changes were required to produce the syndrome in this family (12). After this report, a large number of predominantly heterozygous mutations in *PROK2* and *PROKR2* with considerable clinical and molecular heterogeneity have now been reported in several patients with both KS and nIHH (13–18, 126) (Ta-

TABLE 6. Genotypes/phenotypes of KS and nIHH subjects with *PROKR2* mutations

First author (Ref.)	Patient cohort	No. affected/diagnosis	Mutations	Associated phenotypes
Dode (11)	192 KS	14/KS	2 Homozygous, 2 compound heterozygous, 10 heterozygous	Severe sleep disorder, obesity
Cole (13)	170 KS, 154 nIHH	6/KS, 5/nIHH	All heterozygous	Pes planus, fibrous dysplasia, hearing loss, epilepsy, synkinesia, pectus excavatum
Abreu (16)	63 KS, 44 nIHH	5/KS, 1/nIHH	1 Homozygous, 5 heterozygous	Obesity, metabolic syndrome
Sinisi (14)	1 Patient (case report)	1/KS	Homozygous	None
Canto (17)	24 KS	2/KS	Heterozygous	Atopic dermatitis, abnormal eye movements
Sarfati (18)	Comparative study	25/KS	4 Homozygous, 20 heterozygous, 1 compound heterozygous	High arched palate, type 2 diabetes, nystagmus

bles 5 and 6). The reported *PROK2* mutations and *PROKR2* mutations are depicted in Figs. 2 and 3, respectively.

1. Clinical heterogeneity of PROK2/PROKR2 *mutations*

a. **PROK2** *and* **PROKR2** *mutations cause both KS and nIHH.* Murine homozygous deletions of *prok2* and *prokr2* result in a KS phenocopy. Surprisingly, in humans, mutations in *PROK2* and *PROKR2* cause both KS and nIHH (12, 13, 16) (Tables 5 and 6). The KS phenotype seen in both mice and humans is not surprising, given the role played by prokineticin 2 in olfactory bulb neurogenesis and GnRH neuronal migration during embryonic development. However, the presence of prokineticin 2 pathway mutations in subjects with nIHH suggests an important, yet uncharacterized role for PROK2 in the regulation of GnRH synthesis, secretion, and/or action that is independent of its relationship with olfactory migration. This is also supported by the expression of PROK2 in hypothalamic areas with a "high" density of GnRH neurons. However, given that GnRH neurons in the hypothalamus do not coexpress PROKR2 (Fig. 4) (12), it is presumed that the effect of PROKR2 on GnRH neurons is indirect. Elucidation of the mechanism(s) by which the PROK2 pathway modulates the function of GnRH neurons is currently of significant research interest.

b. Variable expressivity and incomplete penetrance of reproductive and olfactory phenotypes within families. Patients with *PROK2* and *PROKR2* mutations show considerable phenotypic heterogeneity. In general, subjects with homozygous *PROK2* and *PROKR2* mutations exhibit a reproductive phenotype that is often fairly severe and penetrant (12, 16, 18). It is, however, important to remember that the majority of patients with mutations in this pathway harbor heterozygous mutations. In these pedigrees with heterozygous mutations, variable expressivity or incomplete penetrance of both the reproductive and olfactory phenotypes is evident both within and across families sharing identical mutations. For example, whereas some members with a heterozygous mutation show a fully penetrant KS phenotype, others show a wide spectrum of reproductive and olfactory defects such as delayed puberty, normosmic GnRH deficiency, or isolated anosmia, and still others are seemingly unaffected (11, 13). One possible explanation for these observations is that patients with heterozygous mutations in *PROK2/PROKR2* may carry additional genetic mutations in other genes (oligogenicity) that may contribute to the phenotypic presentation (10, 127). Thus, GnRH-deficient subjects with heterozygous mutations in PROK2/PROKR2 represent a unique cohort, and careful genetic investigation is likely to uncover the missing genetic or epigenetic modifiers that interact with this pathway in humans to account for the phenotypic heterogeneity.

c. "Dual" defect and reversal of GnRH deficiency. Although all GnRH-deficient patients with *PROK2/PROKR2* mutations display a hypothalamic defect, in two patients with *PROK2* (15) and *PROKR2* (14) mutations, persistent oligo/azoospermia has been observed during gonadotropin treatment, suggesting a "dual" defect: hypothalamic GnRH deficiency and a primary gonadal defect. The primary gonadal defect in these patients is in keeping with the unique expression profile of PROK2 and PROKR2 in the testes and, in particular, the expression of PROKR2 in primary spermatocytes (63, 81). More recently, in a pilot genome-wide association study, a tagging single nucleotide polymorphism in close proximity to *PROK2* gene has been shown to be associated with oligospermia and azoospermia in men (128). Collectively, these observations also suggest a role for the pro-

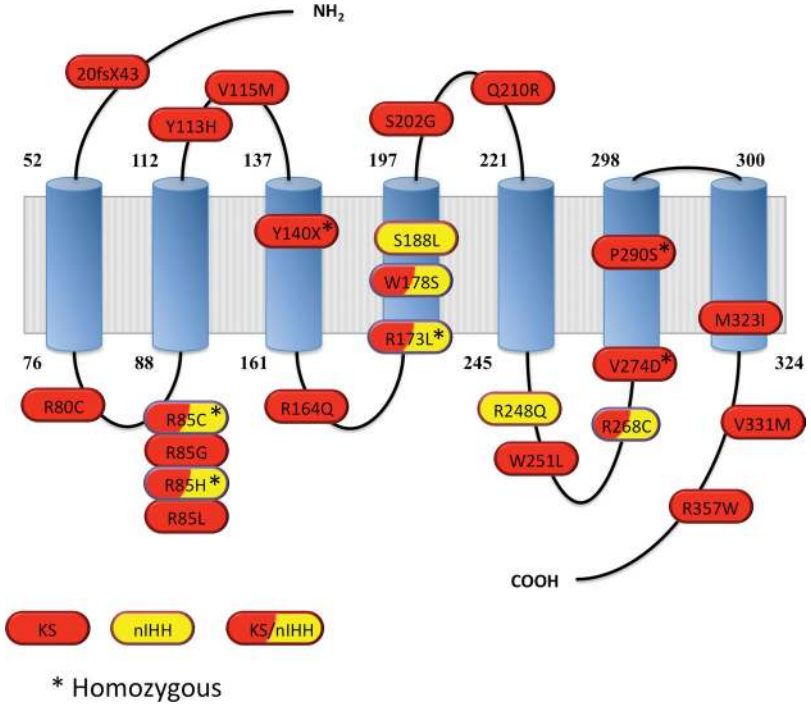

FIG. 3. *PROKR2* gene mutations identified in GnRH-deficient patients. Schematic of the PROKR2 protein generated using the SOSUI secondary structure software (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) showing the seven trans-membrane spans (*blue cylinders*) and the *PROKR2* mutations identified to date in isolated GnRH-deficient patients. Mutations labeled in *red* have been identified in KS patients; mutations labeled in *yellow* have been identified in normosmic GnRH-deficient (nIHH) probands; and *hatched red and yellow label* shows mutations that have been identified in nIHH as well as KS patients (11, 13–18, 135).

kineticin 2 pathway in regulating primary testicular function and spermatogenesis.

Reversal of congenital GnRH deficiency later in adulthood is yet another well recognized phenomenon in patients with both KS and nIHH. Notably, reversal of GnRH deficiency occurs even in the context of deleterious mutations (129). Subjects with mutations in *PROKR2* have also been documented to undergo reversal of GnRH deficiency after treatment with sex steroids (13, 14). Although the precise biological basis for this restoration of GnRH secretion remains unclear, it is likely that GnRH and/or PROK2/PROKR2 neuronal plasticity, possibly modulated by sex steroid treatment and/or other epigenetic effects, may be responsible.

d. Nonreproductive phenotypes of GnRH-deficient patients with PROK2/PROKR2 mutations. GnRH deficiency is often characterized by a number of nonreproductive features (Tables 1 and 2). These clinical and neuroendocrine signatures reflect the expression and function of the underlying causative genes in other organ systems and often provide clues to an unexpected facet of their biology (*e.g.*, renal agenesis and *KAL1* mutations, cleft lip/palate, and *FGFR1* mutations) (52). Among previously reported nonreproductive features of GnRH deficiency, bimanual synkinesia and hearing loss are seen in a minority of patients with *PROK2/PROKR2* mutations, whereas renal agenesis, cleft lip, and cleft palate have not been reported so far (13, 18) (Tables 3 and 4). PROK2 has been proposed as a key candidate linking the reproductive and circadian systems, and both *prok2*$^{-/-}$ and *prokr2*$^{-/-}$ mice exhibit disruptions of some of their circadian rhythms, including abnormal thermogenesis, increased nocturnal physical activity, impaired circadian cortisol secretion, and abnormal glucose regulation (130, 131). In keeping with this notion, some GnRH-deficient patients with mutations in *PROK2/PROKR2* were reported to have sleep disorders (11, 13, 18). However, detailed circadian assessment using a constant routine protocol in two subjects with homozygous *PROK2* mutations did not show any major circadian abnormalities (unpublished data from our group). Similarly, sleep quality and cortisol profile studies in a subset of *PROK2/PROKR2* mutation subjects have also failed to confirm this link in humans (18).

In rodents, PROK2 has been linked to ingestive behavior (132) and hypothalamic appetite regulation (133). Although some subjects with *PROK2/PROKR2* mutations are obese, no consistent evidence of raised body mass index has been documented in these subjects (18). In addition, both *prok2* and *prokr2* knockout mice have been associated with increased neonatal death (12, 113). Although increased neonatal mortality has been seen in a family with *PROK2* mutations in humans (12), the precise role of the prokineticin pathway in neonatal development in humans remains to be determined.

In summary, no specific nonreproductive signature for the prokineticin 2 pathway is currently evident. Considering the widespread tissue expression of PROK2/PROKR2 (Tables 3 and 4), detailed phenotyping of patients with PROK2 pathway mutations and longitudinal follow-up will be essential to unearth any potential links.

2. Genetic heterogeneity: the puzzle of heterozygous mutations

Whereas homozygous deletions of *prok2* and *prokr2* in mice result in hypogonadotropic hypogonadism, heterozygous mice do not show any gross reproductive abnormalities (12, 113), thus suggesting a faithful autosomal recessive mode of inheritance in the mouse. In con-

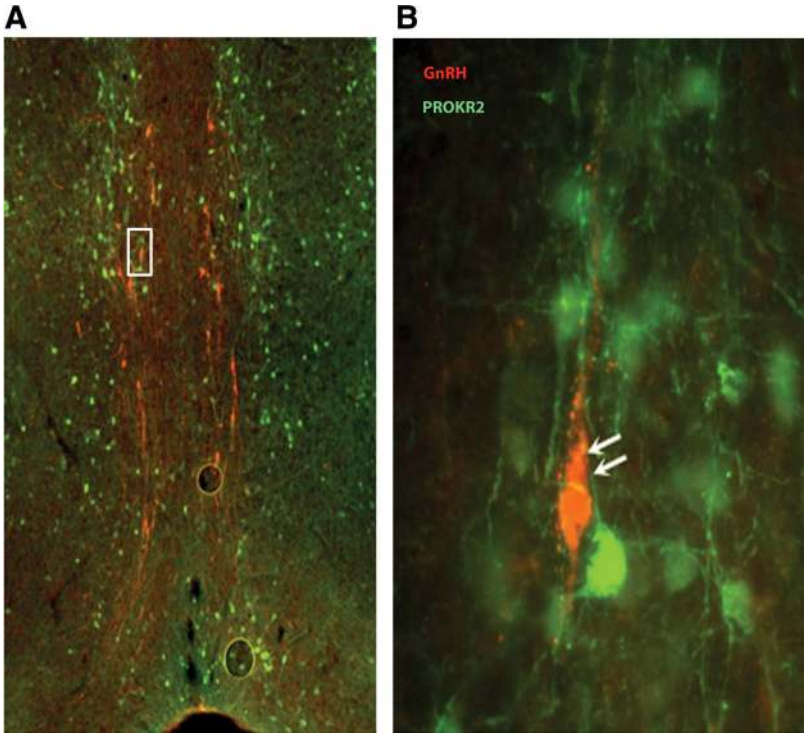

FIG. 4. PROKR2 is not expressed in the GnRH neurons located in the median preoptic area. A, Double immunostaining showing the GnRH neurons (*red*) and PROKR2 immunoreactive cells (*green*) in the preoptic area of PROKR2-GFP transgenic mice. B, A higher magnification view of the boxed area in panel A is shown illustrating the absence of PROKR2 protein expression in GnRH expressing cells.

trast, humans with heterozygous mutations in *PROK2* or *PROKR2* who present with complete isolated GnRH deficiency are puzzling. This anomaly could represent an autosomal dominant mode of inheritance due to either haploinsufficiency or a dominant-negative effect. However, functional studies of selected *PROKR2* mutants to date have failed to show a dominant-negative effect of these mutations (126). Currently, oligogenic inheritance is the most plausible explanation for the phenotypes seen in patients with heterozygous mutations because interaction and synergism between multiple genes causing GnRH deficiency has been demonstrated (10, 15, 134, 135). Accordingly, mutations in genes already linked with GnRH deficiency have been found to be present in some patients with detected heterozygous mutations in *PROK2*/*PROKR2* (11, 13, 17, 18). A comprehensive frequency study of detected protein-altering variants in patients with isolated GnRH deficiency revealed that the likelihood of such oligogenicity was 11% (134). It has been postulated that other nongenetic/environmental modifiers may also contribute to the observed variable phenotypic expression in subjects with heterozygous mutations, although direct evidence for this is still lacking. The potential mechanisms by which heterozygous mutations in PROK2/PROKR2 cause a broad spectrum of reproductive and olfactory phenotypes is shown schematically in Fig. 5. At present, the genetic puzzle of high frequency patients with complete GnRH deficiency who only harbor heterozygous mutations in *PROK2/PROKR2* is of significant research interest and is under active evaluation by our group and by others.

3. Molecular heterogeneity

a. Functional heterogeneity of* PROK2 *and* PROKR2 *mutations. The functional effects of mutations in *PROK2* was first provided by Pitteloud *et al.* (12), with the report of a homozygous deletion in exon 2 of the PROK2 gene resulting in a truncated protein of 27 amino acids rather than the mature form (81 amino acids). After stable expression of the WT PROKR2 in a Chinese hamster ovary (CHO) cell line, an aequorin-based luminescent assay was used to measure intracellular calcium activation by the mutant peptide compared with the mature form. Even at high concentrations, the truncated form of *PROK2* could not activate the receptor, confirming the deleterious nature of the deletion. Similarly, *PROKR2* mutations have also been confirmed to be loss-of-function in transiently transfected cell lines with wild-type and mutant *PROKR2*, and the receptor biology has been functionally assessed in multiple ways: intracellular calcium influx, MAPK activation, protein expression, cell-surface targeting of the receptor, ligand binding, and bioinformatic prediction of function (13, 126).

There is significant heterogeneity in functional impairment of the different *PROK2* and *PROKR2* mutations. Among the *PROK2* mutations, all but the I50M mutation show significant impairment of intracellular calcium influx. In contrast, there is considerable variation in the functional impact of *PROKR2* mutations. Whereas some mutations show significant global impairment of receptor function (L173R, P290S, W178S), others (R85C, R248Q, V331M) preferentially affect the intracellular calcium influx while relatively sparing the MAPK signaling cascade. In contrast, some mutations preferentially affect the MAPK signaling pathway (R357W) (13, 126, 135). The discordant effects of PROKR2 mutations indicate potentially domain-specific effects and will require further evaluation to characterize the structure-activity relationships and identify critical structural elements of the PROKR2.

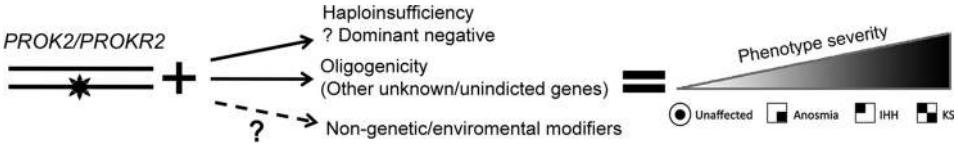

Fig. 5. Variable expressivity and incomplete penetrance in subjects with PROKR2 mutations: representative pedigree and potential mechanisms of how heterozygous PROK2/PROKR2 mutations cause human GnRH deficiency. Schematic representation of potential mechanisms to explain how heterozygous mutations in the PROK2/PROKR2 system cause a broad spectrum of olfactory and reproductive phenotypes. The phenotype severity results from the combination of heterozygous PROK2/PROKR2 mutation along with several factors including haploinsufficiency, oligogenicity, and/or epigenetic modifiers.

VIII. Potential Role of Prokineticin 2 Pathway in Nonreproductive Disorders

Although human GnRH deficiency is the only disease entity currently linked with the prokineticin 2 pathway, both PROK1 and PROK2 play important roles in nonreproductive tissues and multiple physiological functions. This section will briefly review the potential role of the prokineticin 2 pathway in nonreproductive disorders.

A. Nociception and inflammatory pain

Both PROKR1 and PROKR2 are expressed in the dorsal root ganglion, in outer layers of the dorsal horns of the spinal cord and in peripheral terminals of nociceptor axons (136). In rodents, local and systemic injections of very low doses of Bv8 (amphibian homolog of PROK2) decreases nociceptive thresholds to thermal, mechanical, and chemical stimuli through activation of both PROKR1 and PROKR2 in the primary sensory neurons (119). This increase in nociceptor excitability results from functional interaction between PROKR1 and transient receptor potential vanilloid 1, two coexpressing receptors in the dorsal root ganglion. Mice lacking *prok2* and *prokr1* exhibit impaired pain perception to various noxious stimuli (thermal, mechanical, and capsaicin), but preserved tactile sensitivity, suggesting that tactile sensation may signal primarily through PROKR2 (67, 137, 138). Accordingly, mice lacking *prokr2* exhibit lower sensitivity to tactile allodynia while retaining sensitivity to noxious stimuli (67).

Recent studies in rodents also highlight a critical role for PROK2 in granulocyte-mediated inflammatory pain (118, 139). Inflammatory granulocytes and macrophages strongly express both PROK2 and PROK2L isoforms, both of which act as potent pronociceptive agents. In rodent models of inflammatory pain induced by complete Freund's adjuvant, marked up-regulation of PROK2 in granulocytes and macrophages correlates with the development and duration of pain (139). In addition, mice lacking *prokr1* and *prokr2* show significantly less inflammation-induced hyperalgesia, and pretreatment with a nonpeptide PROKR1 antagonist abolishes both hypernociception and inflammatory hyperalgesia induced by PROK2 (139). It remains to be seen whether human subjects with *PROK2* and *PROKR2* mutations show any significant defects in nociception or inflammatory hyperalgesia.

B. Angiogenesis and vascular function of the heart

Until recently, the mammalian heart was considered to be a fully differentiated organ and therefore thought to be unable to regenerate after cardiovascular insult. However, cardiomyocyte-specific overexpression of *prokr1* and *prokr2* in transgenic mouse hearts has highlighted an important role for these receptors in cardiac angiogenesis. Recently, Nebigil and collaborators (140) have shown that transient *prokr1* gene transfer after coronary artery ligation reduces mortality and preserves left ventricular function by promoting new coronary arteriole formation and augmentation of capillary density in the cardiomyocytes. Furthermore, this cardioprotective effect could be explained by an autocrine/paracrine loop where PROKR1 up-regulates PROK2, which in turn induces the reprogramming of adult epicardium resulting in neovascularization after heart injury (141). In sharp contrast, cardiac-specific *prokr2* overexpression in mice leads to dilated cardiomyopathy and induces capillary fenestration and vascular leakage (142). In addition, recent work has also confirmed that PROK2 induces angiogenesis in coronary endothelial cells through PROKR1 activation, whereas PROKR2 activation in coronary endothelial cells results in capillary fenestration (74, 143). Thus, the functional impact of PROK2 on coronary endothelial cells depends on both the expression profile of PROKR1 and PROKR2 and the divergent signaling pathways used by these receptors. The human relevance for these observations is currently unclear.

C. Mood disorders

Disruption of circadian rhythms has previously been linked to mood disorders. In rodents, intracerebroventricular injection of PROK2 results in increased anxiety-like behavior, whereas *prok2* knockout mice display reduced anxiety and depression-like behavior. In a recent case-control study of Japanese patients with mood disorders (151 bipolar patients, 319 major depressive disorder patients, and 340 controls), a tagging single nucleotide polymor-

phism in PROKR2 was significantly associated with major depressive disorder (73). However, considering the small numbers in this report, this observation requires further evaluation in larger samples.

D. Abdominal aortic aneurysm and idiopathic pulmonary arterial hypertension

Prokineticins are potent chemoattractants for monocytes and macrophages both *in vitro* and *in vivo* and stimulate the release of proinflammatory cytokines from macrophages and monocytes. In a human study comparing the whole-genome expression profile from abdominal aortic aneurysm rupture sites and anterior sac biopsies (internal control), *PROK2* emerged as one of 21 differentially expressed genes (144). Similarly, in a small group of patients with idiopathic pulmonary arterial hypertension, PROK2 expression was highly up-regulated in peripheral B cells compared with controls (145). Both of these associations are weak, probably represent a generic role for PROK2 in inflammation, and hence require further validation.

In summary, the role of PROK2 beyond neuronal migration and the observed reproductive and olfactory phenotypes in humans is yet to be determined. Considering the widespread tissue distribution of both PROK2 and PROKR2, detailed phenotyping of human subjects harboring loss-of-function mutations in PROK2/PROKR2 will help to uncover the broader nonreproductive roles of this pathway in humans.

IX. Conclusion

In the last decade, patients with GnRH deficiency have helped us enormously to define the genetic architecture of GnRH neuronal ontogeny in humans. Several key signaling molecules and novel neuropeptides critical for GnRH neuronal development and functional integrity have been discovered. Some of the newly discovered neuropeptides, *e.g.*, kisspeptin, have assumed significant importance and now can be used as additional physiological probes for assessing GnRH neuronal integrity *in vivo* (146, 147). Likewise, the recent discovery of mutations in the prokineticin 2 pathway in human GnRH deficiency has helped to expand our understanding of the complex biology of GnRH neuronal development and function.

Nonetheless, several aspects of prokineticin 2 pathway mutations open up further questions and challenges. In the homozygous state, both the murine and human PROK2/PROKR2 mutations provide compelling evidence for the critical role played by this pathway in embryonic migration of GnRH neurons. However, the presence of *PROK2/PROKR2* mutations in nIHH subjects and the reproductive abnormalities in *prok2* knockout mice with partial olfactory bulb development suggest a potential role for PROK2 beyond GnRH neuronal migration. This fact is particularly interesting given that mature GnRH neurons do not express prokineticin receptors (Fig. 4). At present, the role of other proteins, chaperones, transcription factors, or other second messengers that interact or mediate the molecular effects of PROK2 are unknown and require further detailed investigation.

Careful genetic investigations in humans with extremes of phenotypes have unearthed several key biological insights into physiology and pathogenesis of human disease (148–150). In the same vein, genetic investigations in humans with GnRH deficiency allow us to begin to map the systems biology of GnRH neuronal development and its functional regulation and interactions (167). With expanding genetic and genomic tools and falling sequencing costs, the coming years will be exciting because novel and unexpected candidates involved in GnRH neuronal ontogeny will explode onto the scene. However, defining their biological role and relating them to the known pathways will be extremely challenging and likely to require development of reliable and scalable biological validation systems. Such studies are now imperative so that observations from these unique patient cohorts can be translated to both understand and treat more common reproductive and nonreproductive disorders in humans.

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