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The Puzzles Of the Prokineticin 2 Pathway in Human Reproduction

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Abstract

The Prokineticins, 1 (PROK1) and prokineticin 2 (PROK2), are two closely related proteins that were identified as the mammalian homologues of their two amphibian homologues, mamba intestinal toxin (MIT-1) and Bv8. MIT-1 was initially identified as a non-toxic constituent in the venom of the black mamba snake (*Dendroaspis polylepis*) (Joubert and Strydom 1980) while Bv8 was identified in the skin secretion of the toad, *Bombina variegata* (Mollay, Wechselberger et al. 1999). All three homologues stimulate gastrointestinal motility thus accounting for their family name “prokineticins” (Schweitz, Bidard et al. 1990; Schweitz, Pacaud et al. 1999). However, since its initial description, both PROK1 and PROK2 have been found to regulate a dazzling array of biological functions throughout the body. In particular, PROK1 acts as a potent angiogenic mitogen on endocrine vascular epithelium, thus earning its other name, Endocrine Gland-Vascular Endothelial Factor (EG-VEGF) (LeCouter, Lin et al. 2002). In contrast, the PROK2 signaling pathway is a critical regulator of olfactory bulb morphogenesis and sexual maturation in mammals and this function is the focus of this review.

Keywords

Prokineticin 2; Prokineticin 2 receptor; EG-VEGF; mBv8; GnRH deficiency; GnRH neuronal ontogeny; puberty; Kallmann syndrome; hypogonadotropic hypogonadism

The prokineticin signaling pathway in humans

The mature human PROK1 peptide consists of 86 amino acids and is encoded by a three-exon gene on chromosome 1 (NCBI gene ID: 84432) while the most active PROK2 peptide consists of 81 amino acids and is encoded by a four-exon gene on chromosome 3 (NCBI gene ID: 60675). The additional exon of the PROK2 gene can be alternatively spliced, resulting a longer isoform, PROK2L (102 amino acids) whose function is not well-

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understood (Wechselberger, Puglisi et al. 1999; Chen, Kuei et al. 2005). The ligands, PROK1 and PROK2, show only 45% sequence homology but do share two conserved features essential for their bioactivity: a highly conserved hexapeptide “AVITGA” sequence at their N- terminal and a distinctive structural motif consisting of ten cysteine residues with disulphide cross-linking. Although both PROK1 and PROK2 are co-expressed in various tissues including brain, ovary, testis, placenta, adrenal cortex, peripheral blood cells, intestinal tract, heart, and bone marrow (Ngan and Tam 2008; Negri, Lattanzi et al. 2009), there are some striking differential tissue expression patterns. For example, PROK1 is predominantly expressed in steroidogenic endocrine organs (LeCouter, Kowalski et al. 2001), whereas PROK2 is mainly expressed in the non-steroidogenic cells of the testes and the central nervous system. Also, PROK2 has a distinct rhythmic circadian expression in the suprachiasmatic nucleus and throughout the hypothalamus, key for its reproductive status. (Ferrara, LeCouter et al. 2004; Cheng, Bittman et al. 2005).

Both prokineticins can act as effective ligands for a pair of both G-protein coupled receptors, prokineticin receptor 1 (PROKR1) and 2 (PROKR2). Although the human genes encoding these different receptors are on two different chromosomes (*PROKR1* gene: 2p13.3; *PROKR2* gene: 20p13), the sequences of both receptors are remarkably conserved, displaying nearly 85% identity (Li, Bullock et al. 2001; Masuda, Takatsu et al. 2002; Soga, Matsumoto et al. 2002). Both ligands, PROK1 and the 2 PROK2 isoforms (PROK2 and PROK2L), bind and activate both PROK receptors in nanomolar range although PROK2 has a slightly higher affinity for both receptors (Lin, Bullock et al. 2002; Masuda, Takatsu et al. 2002; Soga, Matsumoto et al. 2002). However, the striking differential anatomical expression patterns of these receptors relates to their diverse biological actions. PROKR2 is abundantly expressed in the brain (olfactory bulb, subventricular zone, preoptic area, the paraventricular nucleus, the arcuate nucleus, and the median eminence) and testes, while PROKR1 is mainly expressed in peripheral tissues such as spleen, prostate, pancreas, heart and blood cells.

PROK2 PATHWAY AND ITS LINK TO REPRODUCTION

(A) *Prok2/Prokr2* knockout mice: The first murine model of Kallmann Syndrome (KS)

Kallmann Syndrome (KS) is a severe neurodevelopmental phenotype resulting from the combined failure of neuronal migration of the olfactory and GnRH neuronal precursors (Seminara, Hayes et al. 1998). Although the *KAL1* gene was the first gene linked to the neurodevelopmental phenotype of KS (see Chapter 2) since the mouse homologue of *kall* has yet to be identified and hence a murine model for KS remained elusive. A prime role for the PROK2/PROKR2 signaling pathway in the neuroendocrine control of mammalian reproduction was thus incidentally discovered when complete murine knockouts of *prok2* and *prokr2* mirrored the human phenotype of Kallmann Syndrome (KS) (Ng, Li et al. 2005; Matsumoto, Yamazaki et al. 2006). Given the initial focus on the primary gastrointestinal role assumed for the prokineticins, this finding was completely unanticipated. However, both *prok2* and *prokr2* knockout mice showed disruption of the neurogenesis of their olfactory bulbs (OB) accompanied by a dramatic reduction of the GnRH-expressing cells in the median preoptic area as well as absence of GnRH neural projections in the median eminence (Pitteloud, Zhang et al. 2007). These findings were a phenocopy of the anatomical observation of KS in humans specifically the arrest of their GnRH neuronal migration. These arrested GnRH neurons in the mouse knock outs forming a ‘fibrocellular mass’ just beyond the cribiform plate immediately prior to their entry into the forebrain (Matsumoto, Yamazaki et al. 2006; Pitteloud, Zhang et al. 2007).

This reduction of hypothalamic GnRH neurons in *prok2* and *prokr2* knockout mice results in failure of GnRH secretion and result in low gonadotropins and impairment of sexual

development and fertility in both male and female mice. Male *prok2* and *prokr2* knockout mice show small seminiferous tubules that lack lumens, absent haploid spermatocytes and spermatids (Pitteloud, Zhang et al. 2007). Similarly, female *prok2* and *prokr2* knockout mice exhibit disrupted estrus cycles as consequence of incomplete follicular development characterized by absence of mature follicles and corpora lutea (Pitteloud, Zhang et al. 2007). Although the reproductive phenotype of the *prok2* and *prokr2* knockout mice are remarkably similar, a major difference between the *prok2* and *prokr2* knockout mice is seen in the olfactory system development. While all *prokr2* knockout mice have a dramatic decrease in the olfactory bulb size (Matsumoto, Yamazaki et al. 2006), only half exhibit an asymmetric olfactory bulb development (Pitteloud, Zhang et al. 2007), suggesting a potential redundancy between the two ligands, PROK1 and PROK2, in the neurogenesis of the OB.

(B) *PROK2* and *PROKR2* mutations in isolated GnRH deficiency in humans

Following these findings in murine knockouts of *prok2* and *prokr2*, the prokineticin 2 pathway became an obvious candidate gene to test for the etiology of human GnRH deficiency. In 2006, Dode *et al* screened 192 unrelated KS patients and reported several DNA sequence changes in both *PROK2* and *PROKR2* without any functional studies in the missense cases (Dode, Teixeira et al. 2006). However, in contrast to the murine knock outs, the majority of these rare sequence variants associated with the clinical phenotype were discovered to exist only in the heterozygous state (four patients with heterozygous mutations in *PROK2* and ten patients with heterozygous *PROKR2* variants). Homozygous or compound heterozygous changes were seen in only 4 subjects. Subsequently, Pitteloud *et al* reported 3 affected siblings with GnRH deficiency (two brothers and one sister of Portuguese ethnicity) all of whom harbored a loss-of-function homozygous deletion in the ligand, *PROK2*, that resulted in a biologically inactive 27 amino acid truncated protein (Pitteloud, Zhang et al. 2007). Following these initial reports, a large number of predominantly heterozygous loss-of-function mutations in both *PROK2* (Fig 1) and *PROKR2* (Fig 2) have now been reported in patients with both KS and nIHH by several independent groups (Sarfati, Guiochon-Mantel et al. ; Abreu, Trarbach et al. 2008; Cole, Sidis et al. 2008; Leroy, Fouveau et al. 2008; Sinisi, Asci et al. 2008; Canto, Munguia et al. 2009; Monnier, Dode et al. 2009). Thus, although both the human and mouse studies have confirmed and firmly established a key role of the PROK2 pathway in mammalian reproduction, several features of this biology remain puzzling, suggesting a more complex systems biology of this pathway in humans. These puzzles are detailed below and the underlying basis for these intriguing observations is yet to be fully ascertained.

The Puzzles of The Prokineticin Pathways

The study of humans with mutations PROK2 pathway have greatly helped expand the initial murine observations. However, strikingly, the combined analysis of the murine and human phenotypes have raised several puzzling observations. First, despite the key neurodevelopmental role of the PROK2 pathway, the PROKR2 receptor is conspicuously absent on both developmental and mature adult GnRH neurons. Second, in contrast to a pure 'neurodevelopmental' phenotype in mice, i.e. a combination of olfactory and reproductive phenotypes, humans with *PROK2/PROKR2* mutations present with both KS as well as normosmic idiopathic hyponadotropic hypogonadism (nIHH). This observation indicates the PROK2 pathway plays a key role in both the neurodevelopmental and neuroendocrine facets of GnRH neuronal ontogeny. Third, while heterozygous gene deletions in mice are reportedly normal, clinical syndromes in humans are predominantly found in the heterozygous state. Fourth, humans with identical *PROK2/PROKR2* mutations show considerable variation in the expressivity and penetrance of both their reproductive and olfactory phenotypes. Fifth, the *in vitro* functional studies of human *PROKR2* mutations

show discordant effects on the various intracellular signaling pathways suggesting unique structure-function relationships. Sixth, a few male subjects with mutations with PROK2 pathway mutations display spermatogenic abnormalities hinting at a potential role for the PROK2 pathway in gonadal function. Finally, evolving links of the PROK2 pathway with other more common non-reproductive disorders suggesting a larger physiologic role of the PROK2 pathway in humans (Fig 3).

PUZZLE 1: GNRH NEURONS DO NOT EXPRESS PROKR2

During development, *PROK2* appears to act as a chemoattractant factor, somehow guiding the olfactory axons towards the olfactory bulb (Ng, Li et al. 2005). The *prok2* and *prokr2* deficient mice confirm this premise as GnRH their neuronal migration are arrested after crossing the cribriform plate where they encounter an olfactory bulb fails to develop. However, studies in wild-type (WT) mice show that despite GnRH and PROKR2 being expressed in similar areas in the brain during embryogenesis and in the adult brain, *in situ* hybridization and double labeling experiments in PROKR2-GFP mice fail to show co-expression of GnRH and PROKR2 both during embryogenesis or in the adult stages of WT mice (Pitteloud, Zhang et al. 2007; Martin, Balasubramanian et al. 2010). In the absence of such co-expression, it remains a mystery as to how the PROK2 system orchestrates the migratory journey of the GnRH neurons. Migrating chains of neuroblasts from the subventricular zone are known to distinctly express PROKR2 (Puverel, Nakatani et al. 2009). Therefore, it is feasible that a hitherto-unknown early neuronal population expressing PROKR2 may govern the migration of the GnRH neuron by virtue of their chemoattractive interaction with the developing OB which shows a high level of PROK2 expression. This hypothesis requires further evaluation.

PUZZLE 2: PROK2/PROKR2 mutations in humans cause both KS and nIHH

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. However, the presence of prokineticin 2 pathway mutations in subjects with nIHH suggests an independent effect of PROK2 pathway on neuroendocrine regulation of GnRH synthesis, secretion and/or action. Interestingly, although only 50% of *prok2* null mice exhibit asymmetric OB morphogenesis, all display sexual immaturity, suggesting additional effects of PROK2 pathway beyond its effects on the neurogenesis of the OB and may account for the isolated loss of GnRH secretion in the absence of olfactory abnormalities. This non-olfactory role of PROK2 in neuroendocrine hormonal regulation is also supported by the localization of PROK2 in hypothalamic regions critical for GnRH functional integrity such as the preoptic area, arcuate nucleus, and median eminence (Cheng, Leslie et al. 2006). PROK2 is also expressed in regions associated with reproductive and feeding behavior such as the islands of Calleja, nucleus accumbens, amygdala and premammillary nucleus (Cheng, Leslie et al. 2006).

Secondly, circadian signals have been proposed to contribute directly to the neuroendocrine control of reproduction and the link between time of day and LH surges in rodents is tightly regulated (de la Iglesia and Schwartz 2006; Ward, Dear et al. 2009). PROK2 is abundantly expressed in a circadian fashion within the suprachiasmatic nucleus (SCN) and PROK2 expressing neurons in the SCN send their neural processes into the preoptic area where the GnRH neurons reside (Cheng, Bullock et al. 2002; Cheng, Leslie et al. 2006; Zhang, Truong et al. 2009). Thus, PROK2 has been viewed as a candidate to modulate GnRH function in adults by acting as a key circadian output molecule from the SCN to mature GnRH neurons (Zhang, Truong et al. 2009). In keeping with this notion, both *prok2* and *prokr2* knock out mice display significant circadian abnormalities. However, circadian data from humans with PROK2 pathway mutations suggest that circadian abnormalities are not a feature in

humans (unpublished observations from our group). Thus, the precise mechanism(s) by which the PROK2 pathway modulates the function of mature GnRH neurons is again a mystery and requires further investigation.

PUZZLE 3: The puzzle of heterozygous mutations

In contrast to the mouse, the vast majority of the PROK2 pathway mutations in humans with isolated GnRH deficiency are heterozygotes. We have previously proposed several hypotheses (Martin, Balasubramanian et al. 2010) to explain this heterozygosity puzzle of the PROK2 pathway mutations including: (i) an autosomal dominant mode of inheritance/haploinsufficiency state; (ii) a dominant-negative effect of the mutations; or (iii) oligogenic interactions with other genes or non-genetic factors. However, an autosomal dominant/haploinsufficiency state has not been supported from the functional studies of selected *PROKR2* mutants to date nor is any dominant-negative effect of these mutations apparent (Monnier, Dode et al. 2009). However, oligogenic interactions with mutations in other genes known to cause GnRH deficiency have been documented in some patients with heterozygous mutations in *PROK2/PROKR2* (Sarfati, Guiochon-Mantel et al.; Dode, Teixeira et al. 2006; Cole, Sidis et al. 2008; Canto, Munguia et al. 2009) and this oligogenicity may well explain part of this puzzle. However, a dominant negative role for the mutations may still be possible and allelic dosing experiments in robust cellular/model organ systems may be required to confirm or refute this hypothesis.

PUZZLE 4: Variable expressivity and incomplete penetrance of reproductive and olfactory phenotypes within families

Subjects with homozygous *PROK2* and *PROKR2* mutations exhibit a reproductive and olfactory phenotype that is typically fairly severe and penetrant (Sarfati, Guiochon-Mantel et al.; Pitteloud, Zhang et al. 2007; Abreu, Trarbach et al. 2008). However, in the heterozygous state, subjects and families with *PROK2* and *PROKR2* mutations also show considerable phenotypic heterogeneity within and across families sharing identical heterozygous mutations, ranging from phenotypically normal individuals to a continuing spectrum of abnormalities that include delayed puberty, normosmic GnRH deficiency, isolated anosmia or a full KS phenotype (Dode, Teixeira et al. 2006; Cole, Sidis et al. 2008). One possible explanation for these observations is that patients with heterozygous mutations in *PROK2/PROKR2* may carry additional genetic mutations in other known or as-yet-unknown genes (oligogenicity) that may modify the phenotypic presentation as has been demonstrated to occur in other genes causing this syndrome (Pitteloud, Quinton et al. 2007). Yet another puzzling feature of the phenotypic variability of patients with Isolated GnRH Deficiency is the reversal in adulthood is now a well recognized phenotypic variant and is known to occur even in patients harboring deleterious mutations (Raivio, Falardeau et al. 2007) including those with mutations in *PROKR2* have also been documented to undergo reversal of GnRH deficiency following treatment with sex steroids (Cole, Sidis et al. 2008; Sinisi, Asci et al. 2008). The precise biological basis for this restoration of GnRH secretion remains unclear; however, it is likely that the neuronal plasticity of the PROK2/PROKR2/GnRH system, possibly modulated by sex steroid treatment and/or other epigenetic effects, may be responsible.

PUZZLE 5: *In vitro* functional heterogeneity of *PROK2* and *PROKR2* mutations

Pitteloud *et al* reported a homozygous deletion in exon 2 of the *PROK2* gene resulting in a truncated protein of 27 amino acids that failed to activate the *PROKR2* receptor even at high concentrations, confirming the deleterious nature of the deletion. Similarly, several of the missense *PROKR2* mutations have been confirmed to be loss-of-function in transiently transfected cell lines with WT and mutant *PROKR2*. However, the intracellular signalling effects of the missense variants show diverse features. The biology of the *PROKR2* receptor

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 (Cole, Sidis et al. 2008; Monnier, Dode et al. 2009). Of the PROKR2 missense variants that have been systematically assessed, some mutations show significant global impairment of receptor function (L173R, P290S, W178S) while others (R85C, R248Q, V331M) preferentially affect either the intracellular calcium influx or the MAPK signalling cascade (R357W) (Cole, Sidis et al. 2008; Monnier, Dode et al. 2009). The discordant effects of *PROKR2* mutations may indicate domain-specific effects and more detailed characterization will allow the mapping of the structure-activity relationships and identify critical structural elements of the PROKR2 receptor. In keeping with this notion, by using a natural mutation affecting the 2nd intracellular loop (R164Q), Peng et al recently demonstrated that the 2nd intracellular loop is critical for Gαq, Gαi and Gα16 and PROKR2 interaction. Similar detailed analysis of the naturally occurring sequence variants is now warranted to map the structure-activity relationship for the PROKR2 receptor.

PUZZLE 6: A Potential “Dual” defect: Hypothalamic and gonadal defects in PROK2 mutations

In addition to the hypothalamic defect in patients harboring PROK2 pathway mutations, in two patients, one with *PROK2* (Leroy, Fouveau et al. 2008) and other with *PROKR2* (Sinisi, Asci et al. 2008) mutation, persistent oligo/azoospermia has been reported despite gonadotropin treatment. These observations suggest the possibility of a “dual” defect: a hypothalamic GnRH deficiency and a primary gonadal defect. The gonadal defect in these patients is in keeping with the expression profile of PROK2 and PROKR2 in the testes and in particular, the expression of PROKR2 in primary spermatocytes (Wechselberger, Puglisi et al. 1999; LeCouter, Lin et al. 2003). Under normal conditions, PROK2 is strongly expressed in diploid spermatocytes (4, 32) that give rise to the haploid spermatocytes after meiotic division. Hence it is possible that PROK2 may play a critical role in final stages of spermatogenesis. More recently, in a pilot genome-wide association study, a tagging SNP in close proximity to *PROK2* gene has been shown to be associated with oligospermia and azoospermia in men (Aston and Carrell 2009). Collectively, these observations also suggest a role for the prokineticin 2 pathway in regulating primary testicular function and spermatogenesis. In contrast, in both women and in female mice with PROK2 deficiency, ovarian function can be restored in response to gonadotropin replacement (15). Taken together, these findings indicate that PROK2 is likely to contribute in spermatogenesis in men, but in females, its predominant role is restricted to its hypothalamic function.

PUZZLE 7: Non-reproductive phenotypes of GnRH deficient patients with PROK2/PROKR2 mutations

Specific genetic mutations in GnRH deficiency is often characterized by characteristic non-reproductive features (e.g, renal agenesis and *KAL-1* mutations, cleft lip/palate and *FGFR1* mutations) (Hardelin and Dode 2008). Amongst previously reported non-reproductive features of GnRH deficiency, bimanual synkinesis and hearing loss has been seen in a minority of patients with *PROK2/PROKR2* mutations whereas renal agenesis, cleft lip and cleft palate have not been reported so far (Sarfati, Guiochon-Mantel et al.; Cole, Sidis et al. 2008). PROK2 has also been proposed as a key candidate linking the reproductive and circadian systems. However, detailed circadian assessment using a constant routine protocol in 2 subjects with homozygous *PROK2* mutations did not show any major circadian abnormalities (unpublished data from our group). Similarly, cortisol profiles and sleep quality studies in a subset of *PROK2/PROKR2* mutation were also reported to be normal (Sarfati, Guiochon-Mantel et al.).

Disruption of circadian rhythms has also been linked to mood disorders. In rodents, intracerebroventricular injection of PROK2 into WT mice results in increased anxiety-like behavior and *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 SNP in *PROKR2* was associated with major depressive disorder (Kishi, Kitajima et al. 2009). No psychiatric symptoms have been reported in GnRH deficient pedigrees with PROK2 pathway mutations. However, this genetic information from human genetic studies of complex traits may well be of some importance.

In rodents, PROK2 has also been linked to ingestive behaviour (Negri, Lattanzi et al. 2004) and hypothalamic appetite regulation (Gardiner, Bataveljic et al.). In humans with PROK2 pathway mutations, no consistent association with obesity or eating disorders has been reported (Sarfati, Guiochon-Mantel et al.). In addition, both *prok2* and *prokr2* knockout mice have been associated with increased neonatal death (Matsumoto, Yamazaki et al. 2006; Pitteloud, Zhang et al. 2007). Although increased neonatal mortality has been seen in a family with PROK2 mutations in humans (Pitteloud, Zhang et al. 2007), this observation has not been reported in other pedigrees. Again, in a human study comparing the whole-genome expression profile from abdominal aortic aneurysm rupture sites and anterior sac biopsies, *PROK2* emerged as one of twenty-one differentially expressed genes (Choke, Cockerill et al. 2009). Similarly, in a small group of patients with idiopathic pulmonary arterial hypertension (IPAH), PROK2 expression was highly up-regulated in peripheral B-cells compared to controls (Ulrich, Taraseviciene-Stewart et al. 2008). The above observations are in keeping with the known hematologic and immunologic role of prokineticins. Prokineticin 2 is a potent chemoattractant for monocytes and macrophages both *in vitro* and *in vivo* and mediate the release of proinflammatory cytokines from macrophages and monocytes. These associations, although suggestive, require further validation.

Conclusions

The puzzles of the prokineticin 2 pathway mutations raise important unanswered questions in our current understanding of the role of this pathway in neuroendocrine regulation of reproduction. The observation that neither developing nor mature GnRH neurons express prokineticin receptors suggests that a significant intermediary pathway may mediate the PROK2 system and the GnRH neuronal network and this remains to be elucidated. The interacting proteins, chaperones, transcription factors or other second messengers that interact or mediate PROK2 signaling are also unknown. The heterozygosity puzzle in *PROK2* and *PROKR2* mutations is also a unique opportunity to map the oligogenic architecture of human GnRH deficiency. Using subjects with heterozygous PROK2 pathway mutations as a form of genetic bait, genome wide approaches including exomic sequencing may help identify as yet unknown interacting genes/epigenetic factors that partner with the PROK2 pathway. Finally, in addition to hypothalamic neurodevelopmental neuroendocrine effects of the PROK2 pathway, additional non-reproductive features in these patients may help unravel the systems biology of the PROK2 pathway beyond the reproductive system.

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Fig 1. *PROK2* mutations in humans with isolated GnRH deficiency
Schematic of *PROK2* gene with identified mutations in humans with isolated GnRH deficiency. Residues from 75–95 is alternatively spliced and is shown in yellow. The “AVITGA” sequence is shown in blue. (Reprinted with permission from Martin C et al, *Endo Reviews*, 2011).

Fig 2. PROKR2 mutations in humans with isolated GnRH deficiency

Schematic of PROKR2 receptor with mutations identified in humans with KS and nIHH. Mutations labeled in red are identified in Kallmann patients, yellow label represent mutations in normosmic GnRH deficient (nIHH) probands, and hatch red and yellow label shows the mutations identified in patients either with nIHH or Kallmann syndrome. Homozygous mutations are marked with (*). (Reprinted with permission from Martin C et al, Endo Reviews, 2011).

Fig 3. Puzzles of Prokineticin pathway mutations in humans with isolated GnRH Deficiency
Human GnRH deficient subjects harboring PROK2 pathway mutations pose several puzzling observations (shown in jigsaws') compared to the murine *prok2/prokr2* pathway mutations. See text for details.