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The role of gonadotropin-releasing hormone neurons in polycystic ovary syndrome

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Abstract: Given the critical central role of gonadotropin-releasing hormone (GnRH) neurons in fertility, it is not surprising the GnRH neural network is implicated in the pathology of polycystic ovary syndrome (PCOS), the most common cause of anovulatory infertility. While many symptoms of PCOS relate most proximately to ovarian dysfunction, the central reproductive neuroendocrine system ultimately drives ovarian function through its regulation of anterior pituitary gonadotropin release. The typical cyclical changes in frequency of GnRH release are often absent in women with PCOS resulting in persistent high-frequency drive that promotes gonadotropin changes—relatively high luteinizing hormone and relatively low follicle-stimulating hormone concentrations—that contribute to ovarian hyperandrogenemia and ovulatory dysfunction. However, the specific mechanisms underpinning GnRH neuron dysfunction in PCOS remain unclear. Here, we summarize several preclinical and clinical studies that explore the causes of aberrant GnRH secretion in PCOS and the role of disordered GnRH secretion in PCOS pathophysiology.

Key words: hyperandrogenemia, gonadotropin-releasing hormone, luteinizing hormone, polycystic ovary syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder seen in the clinic, with prevalence depending on the diagnostic criteria utilized [1]. PCOS affects approximately 10% of women according to the currently-recommended Rotterdam diagnostic criteria, which include evidence of at least two of the following: clinical and/or biochemical hyperandrogenism, chronic oligo- or anovulation, and polycystic ovarian morphology [1-3]. The prevalence of PCOS approximates 6% according to the classic National Institutes of Health (NIH) definition of PCOS, which mandates both hyperandrogenism and ovulatory dysfunction [1, 4]. Finally, PCOS affects approximately 10% of women according to the Androgen Excess and PCOS Society criteria: hyperandrogenism plus either ovulatory dysfunction or polycystic ovarian morphology [1, 5].

PCOS has also been associated with several comorbidities including obesity, insulin resistance and type 2 diabetes, depression and anxiety, obstructive sleep apnea, and endometrial cancer [6-12].

Although the characteristics that define PCOS (i.e., androgen excess, oligo-/anovulation, and polycystic ovarian morphology) are most directly related to ovarian function, the central reproductive neuroendocrine system—the gonadotropin-releasing hormone (GnRH) pulse generator in particular—ultimately drives ovarian function through its regulation of gonadotropin release, and altered GnRH secretion plays a prominent role in the pathophysiology of PCOS.

In this review, we will:

1) detail the clinical evidence supporting GnRH pulse generator dysregulation as a prominent player in the pathophysiology of PCOS;
2) discuss evidence from preclinical animal models of PCOS identifying potential mechanisms underpinning disordered GnRH secretion in PCOS; and
3) review clinical trial data regarding recently-developed pharmacological agents targeting the GnRH neuronal network in the treatment of PCOS.

Neuroendocrine dysfunction in PCOS

The functionally-coordinated assembly of hypothalamic GnRH neurons represent the final node for the neural control of reproductive function. GnRH is secreted in a pulsatile fashion into the hypothalamic portal system, with GnRH pulse frequency largely reflecting the presence and degree of ovarian steroid (progesterone, estradiol) negative feedback. GnRH stimulates pituitary gonadotropes to synthesize and secrete the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Importantly, high and low GnRH pulse frequencies favor LH or FSH secretion, respectively [13, 14]; these effects are largely mediated at the level of gonadotropin gene transcription [15]. Notably, although GnRH release cannot be directly measured in humans, animal studies confirm that each GnRH pulse elicits a secretory burst of LH [16, 17]; thus, patterns of pulsatile GnRH secretion can be inferred from patterns of pulsatile LH secretion in human studies.

Altered gonadotropin secretion in PCOS

The majority of women and adolescents with hyperandrogenic PCOS exhibit increased LH (and by inference GnRH) pulse frequency, increased LH pulse amplitude, and exaggerated LH responses to exogenous GnRH [18-27]. In contrast, such patients demonstrate a relative

deficiency of follicle-stimulating hormone (FSH), as is expected under persistently high-frequency GnRH stimulation [13]. In one study, when excluding those who had recently ovulated, serum LH concentrations and LH-to-FSH ratio were elevated in 75% and 94% of women with PCOS, respectively [24]. Persistently elevated LH pulse frequency and an elevated LH-to-FSH ratio indicate hyperactive GnRH pulse secretion [13], implicating dysfunction of the GnRH pulse generator in PCOS pathology.

The aforementioned abnormalities of gonadotropin secretion materially contribute to the ovarian hyperandrogenemia and ovulatory dysfunction of PCOS. LH is the primary stimulus for ovarian androgen production, and the ovarian hyperandrogenemia of PCOS is clearly LH-dependent. The hyperandrogenemia of PCOS typically does not manifest until after the pubertal increase in LH secretion [28]. In women with PCOS, long-acting GnRH agonists, which suppress gonadotropin secretion, markedly reduce circulating androgen concentrations [29, 30]. Likewise, gonadotropin suppression partly accounts for the efficacy of combined oral contraceptives as a treatment for the hyperandrogenism of PCOS. In addition, relative FSH deficiency limits follicular development, contributing to ovulatory dysfunction in PCOS. These well-documented alterations in gonadotropin secretion in PCOS do not depend on gonadotropin-related genetic variants; nonetheless, the functional importance of such changes is supported by studies associating PCOS with variants in a number of gonadotropin-related genes such as *FSHB* (FSH beta subunit), *FSHR* (FSH receptor), *LHB* (LH beta subunit), and *LHCGR* (LH/choriogonadotropin receptor) [31, 32].

Altered GnRH pulsatility in PCOS

Detailed investigations into the hormonal intricacies of PCOS began to be expanded about four decades ago. Initially, increased LH secretion in PCOS was thought to reflect the positive feedback actions of increased serum estrone concentrations. Exogenous estrone does not, however, appear to alter circulating LH concentrations in women with or without PCOS, forcing investigators to look elsewhere for a mechanism [33].

The central role of altered GnRH secretion in PCOS became apparent after the changing patterns of LH pulses—presumably reflecting changes in pulsatile GnRH release, pulse frequency in particular—were delineated throughout ovulatory cycles [34, 35]. In normal ovulatory menstrual cycles, LH pulse frequency is about one pulse every 90–100 minutes in the early follicular phase and one pulse every 60 minutes in the late follicular phase. Following ovulation, LH pulse frequency falls to about one pulse every 4–6 hours by the mid-luteal phase; this reduction primarily reflects the negative feedback actions of progesterone [35-39].

Progesterone's ability to reduce LH pulse frequency requires the permissive presence of estradiol [40, 41], in line with observations that the progesterone receptor is an estrogen-dependent gene. LH pulse frequency increases again across the luteal-follicular transition, reflecting the loss of inhibition by progesterone, so that by the early follicular phase frequency has increased once again. This increase in GnRH pulse frequency across the luteal-follicular transition is important for the early-follicular increase in FSH secretion that promotes follicular development [42]. The subsequent increase in pulse frequency across the follicular phase—from one pulse every 90–100 minutes to one pulse every 60 minutes—partly accounts for the transition from FSH predominance (early follicular phase) to LH predominance (late follicular phase), as is required for successful ovulatory cycles.

In contrast to these typical cyclic events, both hyperandrogenic adolescents and women with PCOS demonstrate persistently high LH pulse frequency, approximating one pulse per hour, similar to that in the typical late follicular phase [19, 21, 22, 43]. (High LH pulse frequency is a consistent finding in PCOS regardless of obesity status, although obesity per se is associated with relative reductions in mean LH and LH pulse amplitude [24, 25, 44, 45].) Persistently high GnRH pulse frequency is a prominent contributor to the LH excess and relative FSH deficiency of PCOS.

Causes of elevated GnRH pulse frequency in PCOS

Although persistently high LH (GnRH) pulse frequency is an expected consequence of anovulation because of a paucity of progesterone in the circulation, anovulation alone does not explain why many women with PCOS never established regular cycles during puberty. Importantly in this regard, high LH pulse frequency in PCOS also reflects a relative resistance to negative feedback by estradiol and progesterone [46, 47]. Specifically, even when present, progesterone does not suppress LH pulse frequency in women with PCOS to the same extent as women undergoing typical cycles [46, 47]. In one study, seven days of exogenous estradiol and progesterone administration, which produced typical luteal phase levels, reduced LH pulse frequency by 60% in normally-cycling controls, but by only 25% in women with PCOS [47]. Similar findings have been observed in studies of adolescents. LH pulse frequency was increased 25-40% in mid- to late pubertal adolescents with hyperandrogenism compared to pubertal stage-matched controls [22, 26, 48, 49]. Further, some 35-50% of hyperandrogenic adolescents are resistant to suppression of the GnRH pulse generator by combined progesterone/estradiol treatment [48, 49].

GnRH pulse generator resistance to negative feedback restraint in part reflects the central actions of hyperandrogenemia, as feedback suppression can be normalized with androgen-receptor blockade. In a study of adult women with PCOS, pretreatment with the androgen-receptor antagonist flutamide did not alter baseline LH pulse frequency, but it normalized GnRH pulse generator sensitivity to combined progesterone/estradiol negative feedback [50]. Other studies support the hypothesis that hyperandrogenemia *per se* contributes to the development of neuroendocrine abnormalities in PCOS. For example, adolescent girls with congenital adrenal hyperplasia or exaggerated adrenarche may develop LH excess, ovarian hyperandrogenism, and PCOS [51-53]. As described below, prenatally-androgenized (PNA) female monkeys, sheep, and rodents demonstrate increased LH pulse frequency and LH excess [54-56]. Similarly, androgens increase GnRH neuron activity in adult mice [57, 58], and prepubertal testosterone administration can increase post-pubertal LH pulse frequency in female rhesus monkeys [59]. Also of interest in this regard, female rhesus monkeys with naturally higher testosterone levels exhibit higher circulating LH concentrations and LH-to-FSH ratios—findings that again suggest GnRH pulse generator dysfunction in the context of elevated androgens [60]. Together, these findings are consistent with the hypothesis that hyperandrogenemia plays a causative role in PCOS, which is supported by a recent genome-wide association analysis suggesting that higher genetically-determined testosterone levels increase the risk for PCOS [61].

Thus, PCOS appears to be characterized by a vicious cycle in the hypothalamic-pituitary-ovarian axis (Figure 1). Androgen excess, primarily of ovarian origin, impairs GnRH pulse generator sensitivity to negative feedback suppression, leading to persistently high GnRH pulse frequency, which in turn enhances LH secretion and limits FSH secretion, both of which contribute to ovarian hyperandrogenemia and ovulatory dysfunction.

Pubertal genesis of abnormal GnRH secretion in nascent PCOS

Prepubertal children exhibit low-frequency LH pulse frequency, low LH pulse amplitude, and low LH-to-FSH ratio [62]. The onset of puberty is characterized by sleep-associated increases in LH pulse amplitude and frequency [62]. LH pulse frequency while awake gradually increases across puberty, while nocturnal LH pulse frequency changes little across puberty [63, 64]. Thus, in late pubertal girls, LH pulse frequency while awake exceeds sleep-associated LH pulse frequency [63, 64]. Higher GnRH pulse frequencies are presumably important for enhancing LH secretion in pubertal girls, while periods of relatively low GnRH pulse frequency—while awake during early puberty and while asleep in later puberty—may be important for maintaining

adequate FSH secretion. These differential changes in sleep- vs. wake-associated LH pulse frequency across puberty may partly reflect greater sensitivity to progesterone negative feedback on LH release during daytime (vs. nighttime) as described in both early and late pubertal girls [65, 66]. In this regard, McCartney and colleagues proposed a working model regarding the typical maturation of LH/GnRH pulse frequency across puberty [62, 65, 66]: (1) in the state of wakefulness, LH (GnRH) pulse frequency is primarily determined by sex steroid (progesterone) negative feedback, and the GnRH pulse generator is exquisitely sensitive to low progesterone concentrations in early puberty (when androgen concentrations are low); (2) the physiologic and gradual pubertal increase in androgen concentrations antagonizes the negative feedback effects of progesterone, resulting in a gradual increase in waking GnRH pulse frequency; and (3) sleep-associated pulse frequency remains relatively constant across puberty since it is not readily influenced by low (non-luteal) progesterone concentrations.

Neuroendocrine dysfunction appears to be an early finding in some girls at risk of developing PCOS. For example, infant daughters of women with PCOS, who have a 5- to 10-fold increased risk of being diagnosed with adult PCOS [67], demonstrate exaggerated LH responses to acute GnRH agonist stimulation [68]. Daughters of women with PCOS may also exhibit lower serum FSH concentrations during childhood [69], although available studies are not consistent in this regard [28]. Some of the classic neuroendocrine findings of PCOS—elevated basal LH, basal LH-to-FSH ratio, GnRH agonist-stimulated LH, and GnRH agonist-stimulated LH-to-FSH ratio—are not observed in early puberty in daughters of women with PCOS. Studies in Chilean daughters of women with PCOS suggest that these aspects emerge toward the end of puberty (e.g., Tanner stage 4) [28, 70-72]. In addition, in peripubertal girls with hyperandrogenism, increased LH (GnRH) pulse secretion can be detected prior to the onset of menarche [22], suggesting that hyperandrogenemia may modulate the normal evolution of LH (GnRH) secretion across pubertal maturation. However, the mechanisms underlying the emergence of neuroendocrine dysfunction across puberty in those who go on to develop PCOS remain unclear.

Although high LH pulse frequency is independent of obesity, obesity appears to feed into the vicious cycle of hormonal interactions in PCOS and may be an important risk factor for the development of PCOS [73-76]. Peripubertal girls with obesity exhibit 2- to 4-fold elevated serum free testosterone concentrations compared to pubertal stage-matched controls without obesity [77-82]. In such girls, circulating LH concentrations predict elevated free testosterone better than circulating insulin concentrations [79, 83]. As a group, girls with obesity develop elevated

LH pulse frequency by mid- to late puberty [64]. Also of interest, while non-hyperandrogenemic late pubertal girls with obesity exhibit the expected overnight decrease in LH pulse frequency, late pubertal girls with both obesity and hyperandrogenemia demonstrate high-frequency LH pulses during daytime and nighttime hours without the expected overnight decrease [64, 84].

The above evidence supports the hypothesis that androgen excess modulates the pubertal maturation of GnRH secretion, and McCartney and colleagues proposed a working model regarding the pubertal genesis of abnormal GnRH pulse generator function in those with peripubertal hyperandrogenemia. In particular, and in contrast to the typical maturational changes in LH/GnRH pulse frequency across female puberty (described above), when neuroendocrine puberty occurs in the setting of hyperandrogenemia (from any cause), atypically high androgen concentrations markedly antagonize progesterone negative feedback. This causes a rapid transition from low 24-hour GnRH pulse frequency to high 24-hour GnRH pulse frequency—without the prominent sleep-wake changes that may be important for appropriate balance of LH and FSH secretion. High 24-hour GnRH pulse frequency would be expected to cause LH excess and relative FSH deficiency, which would support a progression to full-blown PCOS.

Aberrant reproductive neuroendocrine activity in preclinical animal models with PCOS-like features

Ethical constraints prohibit direct scientific assessment of GnRH release in humans. However, prenatal exposure to androgens programs a number of PCOS-like features in several animal species [85]. For example, in addition to exhibiting ovarian hyperandrogenism and ovulatory dysfunction, prenatally-androgenized (PNA) female monkeys demonstrate central resistance to the negative feedback effects of sex steroids, increased LH (GnRH) pulse frequency, increased circulating LH concentrations, and increased LH-to-FSH ratio [86]. PNA rodents and sheep exhibit similar findings [87, 88]. While PNA mice exhibit PCOS-like neuroendocrine dysfunction, it remains unclear to what degree similar *in vivo* abnormalities (e.g., elevated serum LH, elevated LH pulse frequency) are observed in postnatally-androgenized mice [85, 89]. However, in female monkeys, experimentally producing mild hyperandrogenemia (3.7-fold elevated testosterone concentration) beginning prepubertally produced elevations in post-pubertal LH pulse frequency [59].

We note that the degree to which such animal models are relevant to human PCOS remains controversial, in part because no animal model perfectly replicates any human disorder, including PCOS, and in part because various aspects of reproductive physiology can differ by

species. Further complicating this, PCOS is heterogeneous in its presentation, and different pathogenic factors likely play different relative roles in different subsets of patients. With regard to PNA models, it remains unclear whether women with PCOS were exposed to excess androgens *in utero*. For example, some but not all studies suggest that cord blood androgen concentrations are elevated at the time of delivery in daughters of mothers with PCOS [90]. While direct surveillance of *in utero* androgen exposure is exceedingly difficult in humans, anogenital distance—a surrogate measure of intrauterine androgen exposure—appears to be longer in women with PCOS [91-93], although results are mixed in newborn daughters of mothers with PCOS [94, 95]. Similarly, a recent study suggested that sebum production is temporarily increased in newborn daughters of women with PCOS, consistent with *in utero* exposure to maternal androgen excess [96].

Much of our understanding of the likely neurobiological mechanisms leading to androgen-mediated neuroendocrine dysfunction in PCOS is derived from rodent models. Studies performing electrophysiologic recordings of GnRH neurons in murine brain slices from control vs. dihydrotestosterone (DHT)-treated mice suggest that DHT—a non-aromatizable androgen—increases GnRH neuron firing rates [58]. GnRH neuron firing frequency is similarly increased in adult PNA mice, which have elevated endogenous testosterone production [97, 98]. Consistent with these observations, LH pulse frequency is elevated and relatively resistant to progesterone negative feedback in PNA mice and sheep [55, 99], as it is in women with hyperandrogenic PCOS. Such resistance to progesterone negative feedback in these animal models likely reflects reduced progesterone receptor expression in the arcuate nucleus [100-103].

Interestingly, conditional neuron-specific knockout of the androgen receptor in mice reduces the ability of postnatal DHT administration to induce PCOS-like features such as ovulatory dysfunction, polycystic ovaries, and obesity [104]. These data implicate the importance of neuroendocrine androgen action in the development of PCOS-like features in this model. Also of interest in this regard are mice treated with long-term with the aromatase inhibitor letrozole. These mice exhibit PCOS-like features such as hyperandrogenemia, ovulatory dysfunction, and polycystic ovaries [105]. Many of the neuroendocrine changes observed in letrozole-treated rodents—higher serum LH and lower serum FSH concentrations, reduced progesterone receptor mRNA expression in the mediobasal hypothalamus, higher numbers of arcuate nucleus kisspeptin neurons [105-107]—reflect reduced estrogen negative feedback per se. However, co-treatment with flutamide improves estrous cyclicity and reduces both

hyperandrogenemia and pituitary expression of *Lhb* mRNA, suggesting that some of the neuroendocrine findings in this model likely reflect letrozole-induced hyperandrogenemia [108].

Potential role of γ-aminobutyric acid (GABA)ergic neurons in PCOS-related GnRH neuron dysfunction

The pharmacological agent valproate increases GABAergic tone, and long-term therapeutic use of valproate for epilepsy and bipolar disorder has been associated with an increased risk for PCOS [109, 110]. In addition, cerebrospinal fluid GABA concentrations may be elevated in women with PCOS [111]. Although one study suggested that valproate administration to normal women for one month did not increase LH pulse frequency [112], studies in preclinical animal models suggest that GABAergic neurons play a role in the disordered GnRH secretion characteristic of PCOS.

The influence of sex steroids on GnRH secretion appears to be substantively mediated indirectly through neuronal systems afferent to GnRH neurons. Thus, neuronal circuits afferent to GnRH neurons likely mediate hyperandrogenemia-related GnRH neuron dysfunction in PCOS. Because GnRH neurons have high intracellular chloride concentrations, $GABA_A$-receptor stimulation depolarizes GnRH neurons and can induce action potential firing in these cells [113, 114]. GABAergic transmission to GnRH neurons, as well as the amplitude of the GABAergic postsynaptic currents, is decreased and increased by progesterone and DHT, respectively, suggesting that GABA neurons mediate progesterone-mediated suppression and androgen-mediated stimulation of GnRH neuron activity [88, 115]. In PNA mice, anatomical GABAergic innervation onto GnRH neurons is increased, as is functional excitatory GABAergic drive [102, 116-118]. These GABAergic neurons, originating largely from the arcuate nucleus, demonstrate less colocalization with progesterone receptors compared to control mice, suggesting a possible mechanism for increased GABAergic drive that would potentially be associated with progesterone resistance [102]. Long-term selective activation of arcuate nucleus GABAergic neuron terminals in the rostral preoptic area—where GABAergic terminals densely contact GnRH neurons—leads to a PCOS-like phenotype including hyperandrogenemia and disrupted estrous cycles, along with a possible increase in LH pulse frequency [119]. In addition to influencing GnRH neurons via direct synaptic inputs, GABAergic neurons may influence GnRH release indirectly via arcuate nucleus KNDy neurons. For example, PNA ewes exhibit increased GABAergic appositions onto both mediobasal hypothalamus GnRH neurons and arcuate nucleus KNDy neurons [120]. Overall, these studies imply that PNA causes organizational and functional changes within the GABAergic neuronal networks that in turn

promote GnRH neuron overactivity and LH excess, in addition to other PCOS-like characteristics.

Although the specific mechanisms by which pathological GABA signaling develops remains to be determined, impaired microglia pruning of GABAergic synapses in early development has been implicated [121]. In the PNA mouse model, fewer “sculpting” microglia populate the rostral preoptic area during adolescent development, and microglia in this region are found to engulf fewer GABAergic synapses. Whether prenatal androgen excess directly or indirectly drives changes in microglia behavior remains to be determined, but these data suggest the PNA catalyzes a cascade of events that shape the developing PCOS-like brain prior to disease onset. Also of interest: even though atypically high GABAergic input onto GnRH neurons is observable before puberty and before the emergence of PCOS-like findings in PNA mice [116, 118], both the atypical GABAergic input onto GnRH neurons and the PCOS-like findings can be reversed *after* puberty with androgen-receptor blockade [117, 118].

Potential role of anti-Müllerian hormone in PCOS-related GnRH neuron dysfunction

Serum anti-Müllerian hormone (AMH) concentrations—derived from granulosa cells in preantral and small antral ovarian follicles—are elevated in women with PCOS, including during pregnancy [122, 123]. Another study suggested that cord blood AMH concentrations are elevated in neonates born to women with PCOS [124]. In postmenarchal adolescent daughters of women with PCOS, high circulating LH concentrations correlate with high AMH concentrations [72]. More compellingly, experiments in the mouse model suggest that AMH can directly stimulate GnRH neuron activity and GnRH secretion [122, 125].

Two recent studies indicate that AMH administration to pregnant mice produces a PCOS-like syndrome in female progeny, characterized by increased anogenital distance, disrupted estrous cyclicity, and elevated testosterone concentrations [122, 126]. In one of these studies, female mice born to AMH-treated mothers (PAMH) demonstrated increased mean LH concentrations, LH pulse frequency, GnRH neuron firing rate, and GABAergic appositions onto GnRH neurons [122]. Notably, while AMH appeared to activate GnRH neurons, the fetal effects of maternal AMH administration appeared to reflect GnRH-mediated maternal hyperandrogenism, as AMH did not appear to cross the placental barrier, and maternal cotreatment with a GnRH antagonist prevented the aforementioned manifestations in female offspring [122]. Therefore, elevated AMH in pregnant mothers may contribute to the prenatal androgen excess associated with the development of PCOS features. Also of interest, partial GnRH-receptor inhibition in adult PAMH female mice (i.e., the progeny of pregnant dams treated with AMH) normalized circulating LH

and testosterone concentrations, LH pulse frequency, estrous cyclicity, and ovarian morphology (number of corpora lutea and antral follicles) [122].

In a more-recent study [126], AMH administration to pregnant dams led to hyperandrogenemia, disrupted estrous cyclicity, elevated LH, subfertility, and increased adiposity in first-, second-, and third-generation offspring. Accompanying experiments suggested that transgenerational transmission of epigenetic modifications (DNA hypomethylation) accounted for some but perhaps not all of these findings [126]. For example, treatment of third-generation mice with the methyl donor S-adenosylmethionine normalized ovulatory function, LH, testosterone, and body weight, but it did not appear to reverse an increase in preoptic area *Gnrh1* and *Kiss1* expression observed in PAMH mice [126].

Potential role of kisspeptin neurons in PCOS-related GnRH neuron dysfunction

The neuropeptide kisspeptin potently stimulates GnRH neuron activity and GnRH release. Most arcuate nucleus kisspeptin neurons co-express neurokinin B and dynorphin and have thus been called KNDy (kisspeptin/neurokinin B/dynorphin) neurons. A number of studies suggest that arcuate nucleus KNDy neurons form an extensively-interconnected autoregulatory network, with neurokinin B augmenting and dynorphin reducing KNDy neuron activity [127-130]. Accordingly, arcuate kisspeptin neurons are postulated to be a fundamental component of the GnRH pulse generator [128, 131-133]. In addition, KNDy neurons are believed to at least partly mediate sex steroid negative feedback on GnRH secretion [129, 132, 134].

Women with PCOS appear to have elevated circulating kisspeptin levels (standardized mean difference 1.15 with 95% confidence interval 0.68–1.62) [135]; the source of this kisspeptin is unknown but is unlikely to be the brain. Women with PCOS may be more likely to harbor the GG genotype of the kisspeptin gene polymorphism rs4889 [136]. The relevance of these findings remains uncertain, but they provide initial support for the hypothesis that changes in kisspeptin/KNDy neurons may play a role in the dysregulated GnRH secretion characteristic of PCOS.

Changes in KNDy neurons have been reported in animal models used to study PCOS. PNA rodents exhibit increased arcuate nucleus *Kiss1* expression and/or increased arcuate nucleus kisspeptin neuron numbers in some, but not all, studies [103, 137-139]. PNA rats may also exhibit increased hypothalamic Tac2 (neurokinin B) mRNA expression and increased numbers of arcuate nucleus neurons expressing neurokinin B [137, 139]. In PNA ewes, arcuate nucleus kisspeptin cell body size was increased, but there was no change detected in the numbers of

arcuate nucleus kisspeptin-expressing cells, and fewer arcuate nucleus neurokinin B- and dynorphin-expressing cells were reported [101, 140]. Dynorphin acts through the κ-opioid receptor (KOR), which is expressed on both KNDy neurons and GnRH neurons in ewes [141, 142]—in addition to elsewhere in the brain. The non-selective opioid antagonist naloxone can increase LH release under conditions of progesterone negative feedback suggesting opioids have a restraining effect on the reproductive neuroendocrine system [143]. In one study, PNA mice did not exhibit altered hypothalamic dynorphin mRNA expression, although it is notable that the reproductive parameters of the PNA mice (e.g., estrous cyclicity) were less disrupted in this study [138]. In contrast, a recent study suggested that progesterone-receptor and dynorphin RNA transcript numbers are reduced in the KNDy neurons of PNA mice [103]. Taken together, these studies suggest that the opioid signaling component of the KNDy pathway may be involved in PCOS pathophysiology and may be an important therapeutic target. Indeed, a recent preclinical study in PNA mice suggests that KOR agonist difelikefalin, which does not cross the blood-brain barrier but still has access to regions near the fenestrated capillaries of the median eminence, ameliorates estrous cyclicity in addition to reducing serum testosterone [144]. Such results in an animal model with PCOS-like features suggest that KOR agonists deserve further study as potential pharmacological agents for PCOS.

Recent anatomical evidence suggests that KNDy neurons receive fewer glutamatergic and GABAergic inputs in PNA mice [103]. Interestingly, the firing rates of Tac2-GFP-identified (KNDy) neurons in brain slices from both prepubertal and adult control and PNA mice were unaffected by either age or PNA treatment [145]. This may indicate that the reductions in GABA and glutamate inputs effectively cancel each other out, but further functional studies are required to test this hypothesis. Interestingly, the ability of the neurokinin-3 receptor (NK3R) agonist senktide to increase KNDy neuron firing rate was reduced in 3-week-old PNA mice, implying developmental changes [145]. Of note, the above reports of changes in gene expression could lead to an altered neurosecretory output from KNDy neurons despite similar firing characteristics.

Potential efficacy of pharmacological agents targeting the GnRH-related neuronal network in PCOS

The previously-described data—those suggesting that hyperandrogenemia *per se* causes dysregulated GnRH secretion—implies the potential utility of androgen-receptor blockade in restoring normal GnRH secretion in PCOS. For example, although the androgen-receptor antagonist flutamide did not alter baseline LH pulse frequency in PCOS, it normalized GnRH

pulse generator sensitivity to estradiol and progesterone negative feedback [50]. However, when used in isolation, the overall therapeutic value of androgen-receptor blockade remains unclear. For example, studies are mixed on whether flutamide improves ovulation rates in PCOS [146-148]. In addition, androgen-receptor antagonists may adversely affect the development of male offspring, limiting their therapeutic potential in potentially-fertile women. It remains possible that such agents could have unique benefits during critical developmental windows. For example, a recent retrospective study of adult women with PCOS suggested that, compared to antiandrogen initiation in adulthood, the initiation of antiandrogen treatment during adolescence is associated with a greater likelihood of first childbirth after spontaneous (unassisted) conception during adulthood [149].

Pharmacological agents targeting high-order neuronal control of GnRH secretion (e.g., the KNDy neuronal network) may prove useful in the future. For example, a study in adults with PCOS suggested that the selective NK3R antagonist pavinetant (formerly MLE4901 and AZD4901) administered at a dose of 80 mg/day for one week reduced LH pulse frequency (by 3.55 LH pulses over 8 hours), circulating LH concentrations (50% reduction in LH area under the curve), and basal (i.e., non-pulsatile) LH secretion (80% lower) while preserving FSH secretion [150]. Although the efficacy of NK3R blockade appeared to be diminished over time in this study (i.e., changes were not statistically significant after 28 days of use), the reduction in LH area under the curve, LH-to-FSH ratio, LH pulse frequency, and basal LH secretion remained significantly lower at 28 days when analysis was restricted to non-ovulatory patients [150]. In another study of women with PCOS, 40 mg of pavinetant administered twice daily for 7 days reduced both circulating LH concentrations and LH pulse frequency by nearly 40%, while reducing FSH concentrations by 20% [151]. Although these are interesting proof-of-concept studies, the clinical development of pavinetant has since been abandoned, at least in part because of the potential for liver toxicity [152]. A related, recently-published phase 2a multicenter randomized controlled trial demonstrated that 12 weeks' administration of fezolinetant (ESN364), another NK3R antagonist, at 180 mg/day reduced serum testosterone by approximately 35%, LH and FSH by approximately 60% and 18%, respectively, and LH-to-FSH ratio by nearly 60% [153]. While LH pulse frequency was not assessed in this study, reductions in LH-to-FSH ratio and testosterone were sustained for 12 weeks of treatment [153]. No clear changes in circulating estradiol concentrations or ovulatory function were observed in this relatively short-term study [153]. The success of longer-term NK3R antagonism in PCOS remains to be determined. The potential impact of chronic NK3R antagonism on gonadotropin surge generation and ovulation is unknown. Of note, it is possible that long-term, continuous

NK3R antagonist administration could promote hypogonadotropic hypogonadism, as occurs in some individuals with homozygous loss-of-function variants of *TACR3*, the gene encoding NK3R [154].

The site of action of NK3R antagonists may be the KNDy neuron as discussed above, but it is important to bear in mind that this receptor has also been reported in the terminal regions of GnRH neurons in the rat, and that the NK3R agonist senktide increases GnRH release when applied to the median eminence, even in kisspeptin knock out mice, suggesting that GnRH neurons themselves could also be targeted [155, 156].

Summary and future directions

It has long been recognized that PCOS is associated with persistently high LH (GnRH) pulse frequency and disordered gonadotropin secretion—LH excess and high LH-to-FSH ratio in particular. Although the translational research community has uncovered some of the mechanisms accounting for aberrant GnRH secretion in PCOS, much remains unclear. Hyperandrogenemia *per se* contributes to GnRH pulse generator overactivity, at least in part by reducing GnRH pulse generator sensitivity to sex steroid (progesterone) negative feedback. This leads to persistently high GnRH pulse frequency, which preferentially favors LH production and limits FSH production. In turn, these alterations in gonadotropins bolster ovarian androgen production and contribute to ovulatory dysfunction. The degree to which these alterations of GnRH secretion originate in fetal development remains unclear, but the endogenous androgen excess that develops in prenatally-androgenized animals appears to maintain such abnormalities in prenatally-androgenized animals [50, 98, 117, 118]. In addition, androgen-receptor antagonism normalizes GnRH pulse generator sensitivity to negative feedback in women with PCOS [50] and rescues at least some of the neuroendocrine defects identified in preclinical models [117, 118]. These findings suggest the possibility that androgen-receptor blockade can normalize GnRH secretion in PCOS, although results to date are mixed and additional study is needed. Early studies of agents that modulate GnRH secretion via higher-order neuronal inputs (e.g., selective neurokinin-3 receptor antagonists) also suggest potential promise as future treatments for PCOS. Preclinical models will continue to play an important role in improving our understanding of neuroendocrine dysfunction in PCOS. Future directions should include studies to define the pathogenic neuroendocrine changes occurring during critical developmental windows in addition to the initial testing of novel therapeutics for PCOS.

Figure legend

Figure 1. (A) Simplified model of hypothalamic-pituitary-ovarian interactions during a normal menstrual cycle. (B) Proposed vicious cycle in the hypothalamic-pituitary-ovarian axis in PCOS. [+] = feedforward stimulation; [–] = negative feedback; P4, progesterone.

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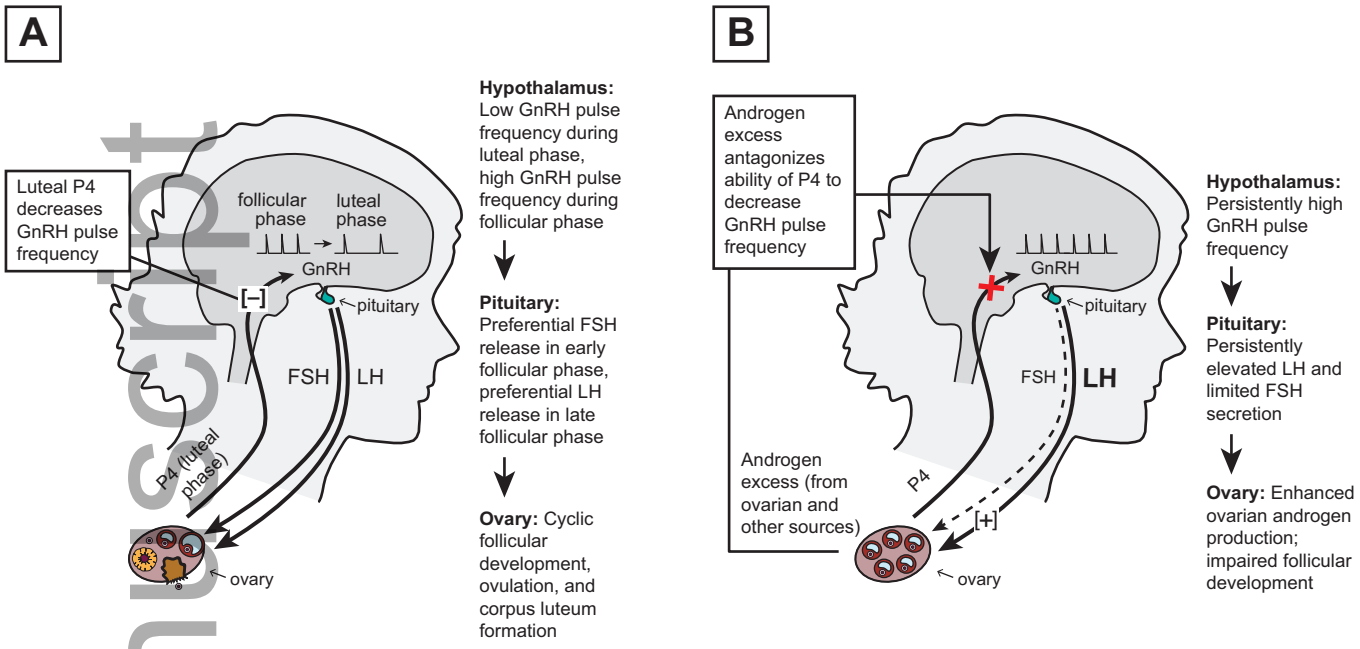
Figure 1



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