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Reproductive neuroendocrine dysfunction in polycystic ovary syndrome: insight from animal models

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Abstract

Polycystic ovary syndrome (PCOS) is a common endocrinopathy with elusive origins. A clinically heterogeneous disorder, PCOS is likely to have multiple etiologies comprised of both genetic and environmental factors. Reproductive neuroendocrine dysfunction involving increased frequency and amplitude of gonadotropin-releasing hormone (GnRH) release, as reflected by pulsatile luteinizing hormone (LH) secretion, is an important pathophysiologic component in PCOS. Whether this defect is primary or secondary to other changes in PCOS is unclear, but it contributes significantly to ongoing reproductive dysfunction. This review highlights recent work in animal models, with a particular emphasis on the mouse, demonstrating the ability of pre- and postnatal steroidal and metabolic factors to drive changes in GnRH/LH pulsatility and GnRH neuron function consistent with the observed abnormalities in PCOS. This work has begun to elucidate how a complex interplay of ovarian, metabolic, and neuroendocrine factors culminates in this syndrome.

Keywords

polycystic ovary syndrome; gonadotropin-releasing hormone neurons; androgens; prenatal androgenization

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common cause of female infertility. The estimated proportion of women affected varies with the criteria used for diagnosis, with the NIH and Rotterdam criteria cited most frequently. NIH criteria, encompassing 6-10% of women (Fauser et al., 2012), are the most strict and require oligo/anovulation and clinical or biochemical signs of hyperandrogenism, with the exclusion of other causes of androgen excess. The Rotterdam consensus workshop defined PCOS as the demonstration of two of three of the following: oligo- or anovulation, hyperandrogenemia or related symptoms such

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as hirsutism, and polycystic ovarian morphology, thereby including patients without hyperandrogenism and expanding the percent of affected women to 15% (Fauser et al., 2012). In addition to these diagnostic phenotypes, PCOS encompasses a range of other common accompanying abnormalities, in particular metabolic disorders (Dunaif, 1997; Lim et al., 2012; Randeva et al., 2012). Common findings are obesity, insulin resistance, abdominal adiposity, and in some cases glucose intolerance, which often progresses to type 2 diabetes mellitus. These metabolic comorbidities raise the risk for cardiovascular disease, a major cause of premature death. PCOS also elevates the risk of endometrial cancer (Dumesic and Lobo, 2013). Added to the distress associated with infertility, the potential health risks of these sequelae reinforce the need for novel preventative strategies and treatments for this disorder.

Despite intense research efforts, the causes of PCOS remain an enigma, due in part to diagnostic inconsistencies, the diversity of clinical phenotypes, as well as the complicated endocrine feedback and feedforward loops involved in the pathophysiology. Existing evidence suggests that PCOS can be caused by both genetic and environmental factors. PCOS clusters in families, implying at least a partial genetic basis. Several genomic variants have been identified as conferring increased susceptibility to PCOS; these include mutations in genes related to insulin resistance, obesity, gonadotropin receptors, and steroidogenesis (Hughes et al., 2006; San Millán et al., 2004). Recently, the association of two single-nucleotide polymorphisms with PCOS was replicated across multiple ethnic populations in genome-wide association studies. Polymorphisms in THADA and DENND1A were associated with endocrine and metabolic disturbances in Han Chinese (Rotterdam criteria; Cui et al., 2013) and European (NIH criteria; Goodarzi et al., 2012) PCOS cohorts. DENND1A was also associated with hyperandrogenemic PCOS in women of European ancestry (NIH criteria; Welt et al., 2012). THADA encodes thyroid adenoma-associated protein and was previously identified as a type 2 diabetes risk locus (Zeggini et al., 2008). The function of the DENDD1A gene is undetermined; it is expressed in androgen-generating theca cells as well as in the brain (Strauss et al., 2012), and potentially may modulate androgen production or GnRH release. Nevertheless, these mutations cannot account for all cases of PCOS. Environmental influences, such as fetal and postnatal diet and hormone exposures, are also likely to be important etiological factors in PCOS.

The more restricted number of patients exhibiting stringent NIH PCOS criteria offers a population with relatively consistent phenotypes for study. In this population, when women who have recently spontaneously ovulated (and hence have elevated serum progesterone) are excluded, most exhibit increased LH pulse frequency (Marshall and Eagleson, 1999), which is determined directly by the GnRH pulse frequency. This indicates that neuroendocrine changes, involving alterations in GnRH neuronal activity, are an important aspect of this disorder.

2. Neuroendocrine dysfunction in PCOS

GnRH neurons are the final central drivers of the reproductive system. A critical feature of these neurons is their ability to secrete GnRH in discrete pulses; the frequency of these pulses is decoded by the pituitary to evoke preferential synthesis and release of LH or FSH

(Wildt et al., 1981). In a healthy woman, the frequency and amplitude of GnRH release undergo cyclical changes across the menstrual cycle in response to steroid feedback (Reame et al., 1984). In the mid follicular phase, estradiol feedback augments GnRH pulse frequency while reducing amplitude (Moenter et al., 1991). The increased frequency drives steroidogenesis, and subsequent rising levels of estradiol provoke a switch in central feedback from negative to positive. This culminates in a surge of GnRH and LH release at mid-cycle that triggers ovulation. Following ovulation, increasing progesterone from the corpus luteum provides negative feedback to reduce GnRH pulsatility. These dynamic changes in the pattern of GnRH release are critical to produce the proper levels of gonadotropins necessary for follicular development and ovulation.

In PCOS, there is substantial evidence for hypothalamic neuroendocrine dysfunction involving disruptions in the pattern of GnRH, and thereby LH and FSH, release. Since GnRH cannot be measured in peripheral serum due to its short half-life and dilution, observations about GnRH release in PCOS are inferred from measures of LH/FSH levels and pulse frequency. Multiple studies have demonstrated increases in LH levels, LH/FSH ratio, and LH pulse frequency and amplitude in women with PCOS (Taylor et al., 1997; Waldstreicher et al., 1988). Downstream at the ovary, the relative suppression of FSH precludes proper follicular maturation, and LH predominance contributes to an overproduction of androgens by theca cells (Gilling-Smith et al., 1994). In sum, these observations indicate that heightened GnRH release, both in frequency and quantity, is an important pathophysiologic aspect in many cases of PCOS. However, whether this defect is primary or secondary to other changes in PCOS remains unclear.

Clinical studies have begun to dissect pharmacologically this abnormal neuroendocrine function in PCOS. One study demonstrated that physiologic levels of circulating progesterone, which are effective to reduce the GnRH/LH pulse frequency in healthy women, failed to reduce the LH pulse frequency in women with PCOS, indicating a failure of normal steroid negative feedback mechanisms (Pastor et al., 1998). In a subsequent study, chronic administration of the androgen antagonist flutamide had no effect on LH pulse frequency or amplitude, LH levels, or response to exogenous GnRH in PCOS patients, indicating that androgen is not a direct cause of these abnormalities (Eagleson et al., 2000). Flutamide did, however, restore the ability of estradiol and progesterone to provide negative central feedback to reduce the LH pulse frequency, suggesting that androgen excess may indirectly contribute to neuroendocrine disruption by interfering with steroid negative feedback.

Clinical studies have also begun to examine the development of PCOS across puberty, when PCOS symptoms are typically first manifested. Hyperandrogenemia in adolescents was found to impede progesterone negative feedback, similar to its action in adult women; however, this effect was only observed in a subpopulation of hyperandrogenemic girls, with progesterone sensitivity being inversely related to fasting insulin levels (Blank et al., 2009; Chhabra et al., 2005). Obese adolescent girls exhibit marked hyperandrogenemia (Coviello et al., 2006; McCartney et al., 2006), as well as elevated free testosterone that is predicted by morning LH and insulin levels (Knudsen et al., 2010; McCartney et al., 2007). Together these findings suggest that peripubertal obesity, by triggering a series of endocrine changes

that lead to a reduction in hypothalamic sensitivity to progesterone, may be an environmental factor contributing to the genesis of PCOS during puberty. Recently, several of these adolescent patients were followed longitudinally; hyperandrogenemia and resultant impaired progesterone feedback were found to progress into late puberty and remain stable thereafter, suggesting that early pubertal disruptions may program lasting changes in neuroendocrine function (Beller et al., 2012).

Despite these advances in understanding the etiology and pathophysiological mechanisms of PCOS, clinical studies face inherent limitations with respect to examining neurobiological changes in this disorder. Manipulation of environmental factors, specifically the prenatal administration of androgens, has allowed the generation of animal models that bear many phenotypic similarities to PCOS in species ranging from rodents to ungulates to primates. Additionally, the development of transgenic mice expressing green fluorescent protein in GnRH neurons has enabled the study of steroidal and metabolic regulation of reproductive function directly at the neuronal level. With these models, several laboratories have begun to dissect the complex interplay of multiple systems that culminates in reproductive neuroendocrine dysfunction in PCOS.

3. Developmental models for the study of PCOS

While no animal model can perfectly recapitulate a human disease, they are invaluable for enabling manipulations not possible in human subjects and isolating physiologic variables to garner insight into disease pathophysiology. Perhaps the best-studied animal models for PCOS are based on prenatal androgen exposure. Early observations in female pseudohermaphrodite monkeys, initially studied for behavioral outcomes (Goy and Resko, 1972), showed that testosterone exposure *in utero* resulted in PCOS-like symptoms such as hyperandrogenemia in adulthood (Abbott et al., 1998), suggesting a developmental etiology for this disorder. This model was further characterized in the monkey and subsequently replicated across several species to yield analogous phenotypes. Women with congenital adrenal hyperplasia, who produce excessive androgens *in utero* that are subsequently normalized after birth, often manifest PCOS symptoms in adulthood, lending etiological relevance to these models (Barnes et al 1994). Women with PCOS exhibit elevated androgen levels during gestation (Maliqueo et al., 2013; Sir-Petermann et al., 2002), and placental tissue from these patients exhibits higher 3β-HSD-1 and lower P450 aromatase activities, which could increase androgen production (Maliqueo et al., 2013). A recent study showed that umbilical vein testosterone in female fetuses of PCOS women is elevated to male levels (Barry et al., 2010), although this has not been a consistent finding (Anderson et al., 2010). The sum of these observations, however, suggests a possible mechanism of excess fetal androgen exposure in PCOS offspring, who are at elevated risk for developing the syndrome.

3.1 Prenatal androgenization recapitulates PCOS phenotypes across species

The ability of prenatal androgen to reproduce both reproductive and metabolic traits of PCOS has been demonstrated in rhesus monkeys, sheep, rats, and mice (reviewed in Abbott et al., 2005; Padmanabhan and Veiga-Lopez, 2013). Rhesus monkeys exposed to testosterone propionate early in gestation (days 40-80 of 165±10 day gestation) develop

anovulation, ovarian hyperandrogenism, enlarged polyfollicular ovaries, and LH hypersecretion, meeting the diagnostic prerequisites for PCOS (Abbott et al., 2002; Dumesic et al., 2002; Eisner et al., 2002). Metabolically, prenatally androgenized (PNA) monkeys exhibit the PCOS characteristics of insulin resistance, hyperlipidemia, glucose intolerance, and increased risk of type 2 diabetes (Abbott et al., 2002; Eisner et al., 2003, 2000). Metabolic dysfunction in PNA monkeys is variable as in human PCOS, and this heterogeneity occurs despite uniform fetal androgen exposure (Abbott et al., 2009), indicating an interaction of genetic influences with the prenatal treatment. In both monkeys and humans, metabolic symptoms are associated with more severe reproductive symptomology (Abbott et al., 2009), illustrating the interaction of multiple physiologic pathways in generating PCOS symptoms.

Similar to monkeys, sheep exposed to testosterone propionate during early- to mid- gestation (GD 30-90 out of 150) develop oligo-ovulation, PCO morphology, LH excess, increased LH pulse frequency, and functional hyperandrogenism (Birch et al., 2003; Manikkam et al., 2008; Ortega et al., 2009; Padmanabhan and Veiga-Lopez, 2011). Hyperandrogenemia in this species results at least in part from changes in the development of ovarian steroid and gonadotropin receptors and steroidogenic enzymes (Hogg et al., 2012, 2011; Ortega et al., 2009) . PNA sheep display multiple metabolic characteristics of PCOS, including excess adult body weight and insulin resistance (Manikkam et al., 2004; Recabarren et al., 2005). Obesity driven by overfeeding was shown to amplify these metabolic and reproductive defects (Steckler et al., 2009). Several studies have parsed out which of the various aspects of this model are programmed by testosterone action at the androgen receptor or through its conversion to estradiol; these data are reviewed elsewhere (Padmanabhan and Veiga-Lopez, 2011). In addition, subgroups of both sheep and monkeys have been assessed following shorter durations of androgen exposure (GD 110-130 in monkey and 60-90 in sheep), which reproduces many of the same symptoms; the materials included here refer to the models with the longer exposures, as these treatments have been used in the majority of studies.

These studies of prenatal androgenization in precocial species have been complemented by work in rodent models. Prenatal testosterone treatment of female rats (d16-19 of 21-23 day gestation) produces reproductive neuroendocrine and metabolic dysfunction, with the severity of the phenotype dependent on the testosterone dose. Lower levels (1 mg/d) of testosterone produce increased LH pulse frequency, while circulating LH and steroid hormone levels are normal; estrous cycle data and ovarian data have not been reported at this dose (Foecking et al., 2005). Higher levels (3 mg/d) of testosterone or DHT cause increased LH and testosterone and reduced FSH, in addition to estrous cycle irregularities and ovarian cysts (Wu et al., 2010). A dose of 5 mg/d prenatal testosterone causes significant metabolic disturbance in this species, marked by elevated body weight, increased adiposity, hyperinsulinemia, and dyslipidemia (Demissie et al., 2008). Interestingly, hyperinsulinemia is not due to impaired insulin sensitivity in this model, and unlike other metabolic manifestations of prenatal androgenization, it is not exacerbated by a high-fat diet (Demissie et al., 2008). The lack of a relationship of insulin with body weight suggests that increased insulin in these animals is a direct result of prenatal androgenization, rather than secondary to increased adiposity. Further, these metabolic effects can also be elicited by

prenatal DHT treatment on the same gestational days, suggesting that the androgen receptor mediates these phenotypes (Yan et al., 2013).

PNA mice are generated by administration of the non-aromatizable androgen DHT late in gestation (d16-18 of 20-21 day gestation). PNA mice exhibit irregular estrous cycles, most often characterized by long durations in diestrus and infrequent proestrus, characteristic of anovulation (Moore et al., 2013; Roland and Moenter, 2011a). Consistent with cycle abnormalities, ovarian morphology is disrupted, showing altered antral follicle morphology and a reduced number of corpora lutea (Moore et al., 2013). LH is elevated, and in some studies mice display elevated testosterone (Sullivan and Moenter, 2004a); this has not been a uniform finding due to the difficulty in performing endocrine measurements in mice and changes in available testosterone assays. There is a tendency for increased anogenital distance in PNA mice but no overt genital virilization, which is not typically observed in PCOS but is found in other PNA models. Anatomical changes in PNA mice did not preclude mating studies, which showed reduced fertility (Moore et al., 2013). PNA mice produce fewer overall litters and reduced pups per litter. Mating behavior and copulatory plugs were not quantified, however, and subfecundity may reflect behavioral defects in addition to anovulation.

Metabolic studies in PNA mice demonstrated elevated fasting glucose, impairments in glucose tolerance and slight changes in other measures such as parametrial (visceral) adipocyte size and adipocyte glucose uptake, but overall reported no marked adiposity, insulin resistance, or abnormal insulin levels (Roland et al., 2010). These findings suggest that the profound metabolic impairments observed in monkey, sheep, and rat PCOS models may be due to differences in the timing of development of metabolic versus neuroendocrine systems among species. The absence of significant metabolic dysfunction or obesity in the context of other PCOS abnormalities would suggest that the PNA mouse serves as a “lean PCOS” model. In some instances this offers an experimental advantage, permitting the study and characterization of neuroendocrine phenotypes without the confounding effects of metabolic disturbances. The observation of the neuroendocrine phenotype of PCOS in the mouse without remarkable metabolic dysfunction is consistent with observations that these disease aspects can be separable in women with PCOS. PCOS encompasses a range of phenotypes, and some women do not display obesity and/or insulin resistance (Diamanti-Kandarakis and Dunaif, 2012), neither of which is a diagnostic prerequisite for the syndrome. However, the data from animal models discussed above indicate that metabolic characteristics, and in particular increasing adiposity, can exacerbate reproductive dysfunction in PCOS (Abbott et al., 2009; Steckler et al., 2009). The ability of insulin sensitizers such as metformin to improve PCOS symptoms supports a causal or at least exacerbating role of metabolic abnormalities (Katsiki et al., 2009). Further, the observation that reproduction is impaired in metabolic conditions such as diabetes and obesity in the absence of elevated androgens (Pasquali et al., 2007) indicates that metabolic factors can have independent in addition to combinatorial effects on reproduction.

The PNA mouse model has been recapitulated in several laboratories using identical prenatal treatments, but resulting in some disparate findings. Two studies showed divergent estrous cycle patterns, with a predominance of either estrus or metestrus (Sullivan and

Moenter, 2004a; Witham et al., 2012). PNA mice exhibited early onset of puberty in two studies, as indicated by the timing of vaginal opening (Roland et al., 2010; Witham et al., 2012), whereas a third study showed delayed puberty marked by later vaginal opening and first estrus (Moore et al., 2013). Fertility reports also differed, with one study showing subfertility over a period of 3 months in adulthood (Moore et al., 2013), and another demonstrating subfertility in the first month of pairing, but subsequent normal fertility (Witham et al., 2012). Impaired glucose tolerance was also not observed in the latter study. The differences among mice generated in different laboratories emphasize that factors other than androgen exposure, such as housing, dietary phytoestrogens, vivarium ventilation, and strain/genetics may be important for the expression of PCOS phenotypes.

3.2 Prenatal androgen programming of steroid feedback

3.2.1 Estrogen feedback—Clinical data (discussed in section 2) have demonstrated that abnormal central steroid feedback plays a fundamental role in the reproductive neuroendocrine dysfunction in PCOS. Abnormal steroid feedback has now been demonstrated in all of the established PNA models. Studies demonstrating the ability of prenatal androgen to masculinize different modes of GnRH secretion by disrupting estradiol feedback have been reviewed previously (Robinson et al., 2002), but we will briefly address these findings. Experiments evaluating steroid feedback in females are typically performed in ovariectomized (OVX) animals to remove the influence of endogenous steroids. This technique distinguishes organizational, permanently programmed effects of steroids from activational effects, which are reversible upon removal of the active steroid. Low-dose estradiol implants that suppress LH levels in OVX control monkeys fail to do so in OVX PNA monkeys, indicating reduced sensitivity to estradiol negative feedback following prenatal androgenization (Steiner et al., 1976). In sheep, estradiol negative feedback was examined in intact animals treated with a GnRH antagonist to reduce endogenous steroids prior to estradiol administration (Sarma et al., 2005). LH levels and pulse frequency were less sensitive to inhibition by estradiol in PNA animals, confirming impaired negative feedback.

In contrast, estradiol positive feedback appears to be differentially regulated in primates compared to other species, with a potentially greater contribution by the pituitary (Karsch et al., 1973; Knobil et al., 1980). This may in part contribute to different outcomes of prenatal androgenization on the LH surge. In rhesus monkeys, the LH surge in response to estradiol benzoate is unchanged following prenatal androgenization of females (Steiner et al., 1976). Further, LH surges in ovary-intact PNA monkeys with regular menstrual cycles are similar to those in untreated females. In sheep, positive feedback was studied in OVX ewes treated with surge-inducing levels of estradiol, resulting in no GnRH or LH surge in PNA ewes in one study (Herbosa et al., 1996), and a delayed LH response in another study (Sharma et al., 2002). This discrepancy may be attributable to the difference in the timing of ovariectomy in these studies, due to the organizational influence of postnatal steroids (Jackson et al., 2013, 2009). Positive feedback has also been assessed in ovary-intact ewes, where control animals showed robust LH surges in response to exogenous estradiol, but the vast majority of PNA animals exhibited no surge at all (Unsworth et al., 2005). Rats prenatally androgenized with either testosterone or DHT also failed to show estradiol benzoate-induced surges (Foecking

et al., 2005). Impairment of induced LH surges in PNA sheep was associated with an absence of normal c-fos activation of GnRH neurons (Wood et al., 1996); interestingly, rhesus monkeys also lack c-fos activation in GnRH neurons despite exhibiting surges (Witkin et al., 1994), further suggesting differential cellular regulation of the surge in this species. Subsequent studies in sheep used a comparison of prenatal testosterone versus DHT to parse out the relative contribution of androgen or estrogen to these programmed alterations in estradiol feedback (Masek et al., 1999; Veiga-Lopez et al., 2009; Wood et al., 1995). In sheep, the effects of prenatal testosterone on estradiol negative feedback were determined to be androgen receptor-dependent, whereas disruptions in estradiol positive feedback were found to be programmed via testosterone conversion to estrogen.

A recent study investigated changes in estradiol feedback and the GnRH surge mechanism in PNA mice, as well as possible developmental changes in neurocircuitry underlying these differences (Moore et al., 2013). Mice were ovariectomized and a subgroup administered estradiol benzoate to examine negative feedback. In uninjected animals, basal LH showed a trend toward an increase in PNA mice, and the post-ovariectomy rise in LH was significantly blunted in PNA mice. Following the estradiol benzoate injection, PNA animals failed to show a normal suppression of LH from post-OVX values, indicating impaired estrogen negative feedback, in accord with other models. Positive feedback was examined using OVX mice given an estradiol implant followed by estradiol benzoate injection. PNA mice demonstrated a robust increase in LH in response to this protocol that did not differ from the response of control mice, indicating intact positive feedback. As these mice were treated *in utero* with DHT, this supports the observation in sheep that disruption of the surge requires estrogen-receptor-dependent mechanisms.

To address potential cellular mechanisms mediating these differences, brain tissue from these mice was subsequently analyzed for c-Fos expression and morphological differences. Expression of c-Fos, an indicator of neuronal activation, was analyzed in GnRH neurons in the rostral preoptic area. GnRH neurons were strongly activated by the estradiol positive feedback paradigm in both control and PNA mice. Spine number on the soma and dendrite of c-Fos-positive and -negative neurons was examined to assess possible differences in the number of afferents to these cells. Spines are sites along the neuron that receive input from single axons. These inputs are typically excitatory (glutamatergic), but inhibitory and excitatory inputs have been observed to synapse onto the same spine (Kubota et al., 2007). Whereas cells from control mice showed an increase in spine density in c-Fos-positive (activated) neurons during the surge compared to cells from oil-treated mice, this difference was not evident in PNA mice (Figure 1; Moore et al., 2013). This may be attributable to the observation that PNA mice had a greater spine density than controls at baseline, and this density was not increased further during the surge. It is interesting that surges occur in PNA mice despite persistent differences in synaptic contacts to GnRH neurons, suggesting that the observed increase in synapses in controls is not a requirement for surge generation and may be more important for driving normal cyclicity. However, anatomical studies cannot provide information on the functional nature of those contacts; functional transmission has been assessed using electrophysiological methods in intact PNA mice and in adult androgen-treatment models (discussed in sections 3.3 and 4.1). Because mice were ovariectomized in

this experiment, the basal increase in synaptic contacts in PNA mice cannot be accounted for by differences in endogenous steroids, and is potentially developmentally programmed. However, as all OVX mice received a low-dose estradiol implant, the difference in synaptic contacts in control and PNA mice may be caused by a differential response to this estradiol.

Along these lines, a previous study demonstrated masculinization of synaptic and glial contacts to GnRH neurons in PNA sheep following ovariectomy and estradiol replacement (Kim et al., 1999). PNA females had reduced synapse density compared to control females. The percent of the neuronal membrane in contact with glia in PNA sheep was intermediate between that of control males and females, with females exhibiting less glial ensheathment. Another recent study extended these findings, demonstrating that prenatal testosterone modifies the synaptic and glial associations with GnRH neurons throughout the course of development (Jansen et al. 2011). In this study, brains were collected from PNA and control sheep at multiple prenatal and postnatal time points (GD90, GD140, 20-23 wk, 10 mo, 21 mo; testosterone administered d30-90). Prior to postnatal brain collection, animals were given two prostaglandin-F2α injections 11 days apart approximately one month prior to killing to induce luteolysis and standardize the hormonal state. PNA treatment was found to cause a slight reduction in GnRH soma size and to alter the number and type of synaptic contacts during both *in utero* development and adulthood. PNA reduced the total number of synaptic contacts to GnRH soma during development (GD140), and reduced contacts to dendrites in adulthood (21 mo), in agreement with Kim et al. (1999). Adding a further level of specificity, GABAergic and glutamatergic inputs were examined by staining for the vesicular transporter proteins VGAT and VGLUT2, respectively; GABAergic inputs to GnRH soma were reduced in PNA sheep at all postnatal ages, and glutamatergic inputs to dendrites were reduced at 10 mo. It is important to point out that VGAT might underestimate GABAergic contacts, as other transporters can load GABA into vesicles. In control sheep there was a tendency to increase synaptic contacts over time, but this increase was absent in PNA sheep. Glial associations with GnRH neurons, measured by GFAP staining, were reduced by PNA at 21 months of age. These experiments provide evidence for marked alterations in synaptic contacts to GnRH neurons throughout development as well as in adulthood in PNA sheep; this, along with altered glial associations, may contribute to disrupted neuroendocrine function in these animals. Interestingly, these data contrast with the above findings from mice, in which spines were increased in the PNA group. Differences in the prenatal steroid, treatment of the animals prior to killing, subregion analyzed (mouse was POA-specific), and potential species differences in synaptic regulation of GnRH neurons may contribute to the divergence of these findings.

3.2.2 Progesterone feedback—Disrupted progesterone negative feedback has been demonstrated in both PNA monkeys and sheep (Levine et al., 2005; Robinson et al., 1999; Veiga-Lopez et al., 2008). A recent study in sheep examined the potential role of kisspeptin/neurokinin B/dynorphin (KNDy) neurons in the arcuate nucleus in driving this impaired progesterone feedback (Cheng et al., 2010). This particular neuronal subpopulation co-expresses three different neuropeptides that can differentially affect GnRH release: dynorphin inhibits, whereas neurokinin B and kisspeptin activate, GnRH neurons (Lehman et al., 2010). KNDy neurons were previously shown to mediate progesterone negative

feedback on GnRH release (Goodman et al., 2004). Prenatal testosterone treatment did not alter the number of kisspeptin-positive cells in ewes, whereas the number of cells expressing either dynorphin, neurokinin B, or the progesterone receptor was reduced. Percent colocalization of any neuropeptide with the progesterone receptor was unchanged by prenatal testosterone; however, the number of cells singly labeled for kisspeptin was increased in the PNA group. This suggests a loss of dynorphin/neurokinin B from kisspeptin cells that would normally colocalize these peptides. These results support the finding that PNA can reduce hypothalamic progesterone receptor expression. The authors further propose that the loss of a neuropeptide that inhibits GnRH release, combined with preserved kisspeptin expression, results in a loss of the net inhibitory effect of this cell population in response to progesterone.

The PNA rat model was used to study changes in hypothalamic progesterone receptor (PR) expression following prenatal androgenization (Foecking et al., 2005). This group previously showed that estradiol-induced PR expression is critical for generation of the GnRH surge in mice (Chappell et al., 1999), and thus proposed that masculinization of the hypothalamus by prenatal androgen renders female rats incapable of surge generation in part by blunting PR expression. In PNA rats, progesterone receptor A+B (assessed in a single measurement) expression in the mediobasal hypothalamus and POA was reduced on proestrus, corresponding with the time of the GnRH/LH surge. Estradiol benzoate (EB) administration to ovariectomized rats doubled PR A+B expression in control, but not prenatal testosterone- or DHT-treated rats, consistent with their inability to produce surges with this paradigm. There was no detectable increase in the pituitary response to exogenous GnRH in either PNA group, suggesting that differences in LH secretion were centrally mediated. These findings were confirmed by another group (Wu et al., 2010). These data suggest that impaired progesterone negative feedback, increased GnRH pulse frequency, and refractoriness to surge generation in PNA animals may be driven in part by reduced hypothalamic progesterone receptor expression. Although serum testosterone was not elevated in this particular rat model, experimental hyperandrogenemia was found to suppress hypothalamic PR expression in adult female rats in a subsequent study, suggesting that activational effects of androgens may exacerbate these programmed changes (Foecking and Levine, 2005).

3.3 Prenatal androgen effects on GnRH neuronal activity

Perhaps the most important advantage of modeling PCOS phenotypes in mice is the ability for genetic manipulation not possible in larger species. This PNA model was originally generated on a GnRH-GFP transgenic background to allow direct targeting of GnRH neurons for electrophysiological recordings (Suter et al., 2000). These recordings are not performed *in vivo*; rather, brains are removed, sectioned, and maintained alive for several hours by continuous perifusion of oxygenated artificial cerebral spinal fluid. While this technique cannot preserve all synaptic contacts and native levels of circulating nutrients and hormones, it has proven useful for studying the effects of *in vivo* exposure to steroid hormones on the brain. Recordings from GnRH neurons in brain slices made at different times of day have been shown to correspond with daily variances in LH levels, demonstrating that activity measures in slices are concordant with native GnRH neuronal

activity (Christian et al., 2005). Further, recent advancements have allowed quantification of GnRH secretion within the brain slice. Hormonal treatments previously shown to produce elevated GnRH neuron activity also increased GnRH release in separate experiments in the same animal model, suggesting correlation between these measures (Glanowska et al., 2012).

The first electrophysiological study using PNA mice examined GABAergic neurotransmission to GnRH neurons (Sullivan and Moenter, 2004a). This was studied in mice in diestrus, the least hormonally dynamic phase of the estrous cycle. GABA has a major presynaptic influence on GnRH neurons and participates in communicating steroidal and metabolic feedback. Previously, there was some controversy about the effect of $GABA_A$ receptor activation on GnRH neurons. The current consensus is that this effect is excitatory (Herbison and Moenter, 2011). Relatively high intracellular chloride maintained by the vast majority of GnRH neurons alters the gradient for ion flow through the chloride channel of the $GABA_A$ receptor, so that when transmitter binds, depolarizing currents are generated at physiologic membrane potentials. These currents are sufficiently large to generate action potential firing when GABA is locally applied to GnRH neurons (DeFazio et al., 2002). Consistent with an excitatory effect of GABA on GnRH neurons, GABAergic neurotransmission to GnRH neurons in PNA mice was increased, as demonstrated by increased postsynaptic current frequency and amplitude. Increased frequency of postsynaptic currents can be due to either increased activity of the afferent GABAergic neuron or altered synaptic connectivity. These changes were shown to be independent of afferent neuronal activity, as the differences persisted when action potentials were blocked by *in vitro* tetrodotoxin. This suggests an increased number of GABA release sites on GnRH neurons. Changes in GABAergic transmission were reversed by *in vivo* treatment for 5-10 d with the androgen receptor blocker flutamide, suggesting that increased GABA neurotransmission was caused by the elevated androgen in adulthood in these mice (Figure 2; Sullivan and Moenter, 2004a). Anatomic studies discussed in section 3.2.1 showed an increased spine number on GnRH neurons in OVX PNA mice that was insensitive to estradiol; it would be interesting to assess this further with respect to the effects of androgens on synapse number, and to further examine GABAergic synapses specifically.

Given an excitatory effect GABA, increased GABAergic transmission suggested that GnRH neuron activity would be increased in PNA mice. GnRH neuron firing activity was examined directly in a subsequent inquiry (Roland and Moenter, 2011a). GnRH neurons in brain slices from PNA mice exhibited an increased mean firing frequency and shorter intervals of quiescence (periods of little to no firing activity that may represent inter-pulse intervals). This finding is consistent with, although it does not directly demonstrate, a pattern of elevated GnRH release, and it also coincides with the elevated LH levels observed in this model. Combined with the previous study, one possible interpretation for these data is that increased firing frequency in GnRH neurons in PNA mice is due at least in part to increased input from GABAergic afferent neurons, as well as increased responsiveness of the GnRH neuron to $GABA_A$ receptor activation.

This study also examined estrous cyclicity and GnRH neuron firing activity in PNA mice following chronic (>10 wk) treatment with metformin. Metformin is commonly used in the

treatment of PCOS due to its insulin-sensitizing properties, which are mediated by activation of AMPK. In women with PCOS, metformin reduces insulin and androgen levels and improves menstrual cyclicity (Palomba et al., 2009). It also has been shown in multiple studies to reduce the LH pulse amplitude in non-obese PCOS patients (Genazzani et al., 2006, 2004), suggesting a possible reduction in GnRH release. At baseline, PNA mice lacked measurable defects in insulin sensitivity, as measured by insulin tolerance testing, or in fed glucose or insulin levels, but tended toward elevated fasting glucose. Metformin did not change these parameters in control or PNA mice, with the exception of reducing fasting glucose levels in PNA mice, demonstrating an effective dose. Metformin markedly improved estrous cycles and restored LH levels to normal in PNA mice. Mechanistically, this improvement was associated with a reduction in the mean firing frequency of GnRH neurons in brain slices from these animals (Figure 3; Roland and Moenter, 2011a). Testosterone and androstenedione levels were not reduced by metformin, suggesting that a reduction in androgens did not mediate these changes. Although the expression of AMPK in GnRH neurons has not been established, the AMPK antagonist compound C (CC) was applied acutely to brain slices to assess the possibility that metformin treatment activated central AMPK (thus enabling its antagonism). CC elicited robust firing responses from GnRH neurons, but only in cells from those mice (both control and PNA) that had been treated with metformin. Additionally, GnRH neurons from metformin-treated mice were relatively insensitive to changes in extracellular glucose, a response dependent on AMPK (see section 4.2). Together these experiments suggested a novel mechanism of action of metformin in improving reproductive cyclicity. In addition to acting indirectly to reduce insulin and thereby androgen levels in women with PCOS, as well as having potential pituitary and ovarian effects, metformin may act directly in the brain to reduce excessive GnRH release. It should be noted that these effects were not proven to be direct on GnRH neurons; other upstream neurons, or potentially glial cells, may deliver these signals to GnRH neurons.

An interesting question with regard to PNA treatments, particularly with aromatizable androgens, is whether the brain of androgenized females is reprogrammed by androgens and/or estrogens during the perinatal sensitive period. The experiments described above (Section 3.2) in ovariectomized animals have demonstrated that both estrogen and androgen play a role in masculinizing the GnRH pulse generator, but they do so by programming different aspects of its function (Masek et al., 1999; Veiga-Lopez et al., 2009; Wood et al., 1995). The prenatal DHT-treated mouse model indeed differs distinctly from most other models due to lack of estradiol-induced programming effects, which in sheep and rats disrupts estradiol positive feedback and the GnRH/LH surge. It is an open question whether or not this estrogenic programing is acting via the same mechanisms that have been shown to be involved in masculinizing the surge-generating apparatus (Handa and Gorski, 1985; Simerly et al., 1985). Despite a lack of change in estradiol-induced LH surges, PNA mice are likely to exhibit changes in pulsatile GnRH release, which have been demonstrated to be dependent on prenatal androgen receptor activation in other PNA models. As the electrophysiological studies in PNA mice have been performed in intact animals, there remains the question of whether the abnormalities observed in these studies are due to programming effects of DHT on the GnRH pulse generator, or to altered steroid feedback.

Examination of GnRH neuronal activity in these mice following ovariectomy will be a goal of future studies to establish central organizational effects.

Despite the absence of programming effects of estradiol, PNA mice exhibit the majority of neuroendocrine PCOS phenotypes, indicating that androgen receptor activation is sufficient to produce a PCOS-like syndrome, at least in mice. While the use of DHT treatment *in utero* simplifies the scientific interpretation, earlier models have used testosterone to mimic the potential exposure proposed to occur in a human fetus whose mother has elevated androgens (as occurs in women with PCOS (Sir-Petermann et al., 2002)). This does not invalidate the use of DHT alone, as this work has shown that the androgen receptor mediates many, if not most, of the deleterious effects of prenatal testosterone excess on the reproductive system. Further, programming effects of DHT are highly relevant to recent work on endocrine disruptors, as several endocrine disrupting compounds can activate the androgen receptor (Gray et al., 2006).

3.4 Early-life antecedents of PCOS in PNA models

With the reproductive and metabolic outcomes of prenatal androgenization clearly established, recent studies have begun to examine changes that occur within the maternal and fetal compartments during pregnancy, and in offspring in early life, that precede the manifestation of PCOS-like symptoms in adulthood. A recent study in rhesus monkeys examined how prenatal testosterone alters levels of reproductive hormones in PNA offspring across fetal and neonatal time points (Abbott et al., 2008). Although a proportion of maternally injected testosterone was aromatized by the placenta, testosterone and androstenedione in female fetuses were elevated to fetal male levels at the time of testosterone treatment (GD 40-80). Androstenedione, but not testosterone, remained elevated in the monkeys as infants. Between days 80-120 of gestation, bioactive LH remained constant in control fetuses; in contrast, PNA fetuses had LH that was initially suppressed to undetectable levels by the testosterone injection and subsequently rose across gestation so that by GD 120, LH levels were higher in PNA than controls. This increase persisted in neonatal monkeys assessed at postnatal days 1 and 30. PNA fetuses and infants also exhibited a higher LH response to an exogenous GnRH injection, indicating that increased LH may in part result from increased pituitary responsiveness to GnRH. These data suggest that abnormal central and/or pituitary programming by prenatal androgen changes the trajectory of LH secretion beginning *in utero*. Such early changes in hypothalamo-pituitary input may subsequently alter ovarian development.

Another study examined metabolic disruptions, both maternal and fetal, occurring during gestation in rhesus monkeys following testosterone treatment (Abbott et al., 2010). In dams, prenatal testosterone treatment caused a transient acceleration in weight gain, increased basal insulin levels, and impaired glucose tolerance during gestation. Female PNA offspring showed accelerated head growth during gestation, consistent with the observed changes in maternal glucose processing, and hyperglycemia and insulin hypersecretion shortly after birth. This suggests that some outcomes of prenatal androgenization are potentially mediated indirectly by metabolic changes in the mother and fetus *in utero*. Supporting the role of programming by PNA-induced metabolic changes is the observation that PNA sheep exhibit

intrauterine growth restriction and early postnatal catch-up growth (Manikkam et al., 2004). This developmental pattern has been causally associated with adverse metabolic consequences (Morrison et al., 2010). Further, women who were born small for gestational age are at increased risk for PCOS (de Zegher and Ibáñez, 2006; Longo et al., 2013; Melo et al., 2010). In daughters of women with PCOS, contradictory outcomes have been observed with respect to birth weight, with some studies showing intrauterine growth restriction (Kjerulff et al., 2011; Sir-Petermann et al., 2005), and at least one study demonstrating a high prevalence of large for gestational age births (Anderson et al., 2010). These outcomes may be dependent on the metabolic health of the mother, as maternal adiposity is a strong predictor of offspring birth weight, and the degree of metabolic dysfunction in PCOS is highly variable. In any case, it remains of interest what role these early metabolic perturbations play in potentially reprogramming the reproductive axis. A recent study found no effect of prenatal treatment with the insulin sensitizer rosiglitazone on PNA-induced disruptions in estradiol negative and positive feedback (Abi Salloum et al., 2012); however, GnRH/LH pulsatility in intact animals and other aspects of neuroendocrine function remain to be studied following this and similar prenatal interventions.

Precocious puberty is a risk factor for PCOS (Ibáñez et al., 2001, 1998), although not all women with PCOS experience this condition. Interestingly, girls exhibiting the combination of low birth weight followed by precocious pubarche are at particular risk for the development of PCOS, and the progression to PCOS in these individuals can be ameliorated by chronic metformin treatment during puberty (Ibáñez et al., 2011, 2004). In multiple PNA models, advanced puberty is a precursor to the development of abnormal reproductive cycles. One study reported normal timing of puberty in PNA sheep, as determined by the start of the first progestogenic cycle (Sharma et al., 2002), while another reported advanced puberty marked by a precocious rise in LH in gonad-intact lambs (Birch et al., 2003). This early LH rise reflects a premature reduction in sensitivity to estradiol feedback in PNA sheep, which was shown to be dependent on the androgen receptor-mediated actions of prenatal testosterone (Masek et al., 1999). The ability of prenatal testosterone to advance puberty is dose-dependent, with higher testosterone doses producing earlier pubertal LH increases (Kosut et al., 1997). PNA mice have also exhibited early onset of puberty in two studies, as indicated by the timing of vaginal opening (Roland et al., 2010; Witham et al., 2012), whereas a third study showed delayed puberty marked by later vaginal opening and first estrus (Moore et al., 2013).

Pubertal advancement in PNA animals appears to be in part mediated by organizational effects of androgens on the neuroendocrine system, as precocious LH pulses can be detected even in prepubertal PNA lambs that have been ovariectomized (Jackson et al., 2009; Wood et al., 1995, 1991). Androgens may also have activational effects to alter the timing of puberty. A recent study in mice demonstrated that androgen receptor activation plays a role in regulating the timing of pubertal onset in females (Brill and Moenter, 2009). In this study, chronic androgen receptor blockade beginning at weaning delayed vaginal opening in control females and reversed the advancement of vaginal opening by high-fat diet. Another recent mouse study (Witham et al., 2012) administered low-dose DHT to female mice at the onset of the pubertal window, on postnatal days 21-23. DHT accelerated the onset of vaginal opening, without an effect on body weight. This experiment was repeated in mice lacking

neuronal androgen receptors, demonstrating a similar advancement of vaginal opening, indicating that the effect of androgen to advance puberty is not mediated directly at the neuronal level. This suggests a peripheral intermediary between androgen and GnRH neurons. Alternatively, androgen may be acting peripherally to alter vaginal opening without accompanying GnRH neuronal activation, as other measures of pubertal onset were not recorded. Central puberty should be confirmed following this treatment. These findings suggest that prepubertal hyperandrogenemia, which may be present in PNA models, may contribute to a premature reduction in hypothalamic sensitivity to steroid feedback and subsequent advanced puberty. Androgens may achieve this indirectly by altering levels of other peripheral hormones.

This and other recent studies have also examined the possibility that puberty may be an additional critical period in which the neuroendocrine system is susceptible to reprogramming by environmental influences, and androgens in particular. Mice that were given prepubertal DHT injections were followed into adulthood to determine if estrous cyclicity was permanently affected; no effect was observed (Witham et al., 2012). These mice may express other reproductive phenotypes that were not examined. Another study assessed the consequences of experimental hyperandrogenemia during puberty in rhesus monkeys (McGee et al., 2012). Administration of a mild elevation of testosterone during the peripubertal period (age 1 through 5.5 y) did not change the timing of pubertal onset. At age 5, however, testosterone-treated monkeys had elevated body weight, increased LH pulse frequency, and greater LH response to GnRH injection, but normal menstrual cycles. It is unclear whether any of the abnormalities observed in monkeys would persist following removal of the exogenous testosterone; this remains a question of interest. However, these findings support recent observations in peripubertal girls that elevated androgens during puberty cause increased GnRH pulsatility, which in girls appears to be a persistent phenomenon (Beller et al., 2012).

4. Adult treatment models for the study of PCOS

4.1 Steroid modulation of GnRH neuron function

In addition to prenatal treatment models, adult treatment models have been used to examine how activational effects of androgens and other factors independently affect GnRH neurons that have undergone normal development. Understanding these effects is indispensible for interpreting data regarding the contributions of endocrine and metabolic changes subsequent to prenatal androgenization. Androgens are normally found at low levels in adult females and are thought to play physiological roles in regulating reproductive cyclicity (Foecking et al., 2008). However, at high levels, androgens can have pathophysiological effects on the female reproductive axis. Studies performed in rats have examined in some depth the effect of elevated androgens on GnRH/LH release in females. Male-typical doses of T or DHT suppress GnRH-induced LH secretion and block estradiol benzoate-induced LH surges in rats (Foecking and Levine, 2005). The latter is achieved at least partly through a suppression of hypothalamic progesterone receptor expression (Foecking and Levine, 2005), which is a prerequisite for these surges (Chappell and Levine, 2000). These studies have been reviewed in detail previously (Foecking et al., 2008). Here we will focus on electrophysiology studies

performed in mice that reveal pathophysiological actions of androgen in females at the neuronal level.

One such study employed treatment of ovariectomized adult female mice with steroid implants, followed by electrophysiological recordings of brain slices from these mice, to determine how different steroid milieux regulate the firing patterns of GnRH neurons (Pielecka et al., 2006). Mice were treated with estradiol and either progesterone, DHT, or both. Progesterone inhibited GnRH neuron firing activity, as shown by decreases in mean firing rate and increases in duration of quiescence. Conversely, DHT stimulated firing activity, demonstrated by increased mean firing rate, reduced quiescent time, and increased algorithmically identified peaks of activity that are postulated to correlate with GnRH pulses (Figure 4; Pielecka et al., 2006). DHT exerted these effects both independently, and by rendering progesterone ineffective to suppress firing activity. This latter finding mirrored the observation in women with PCOS that androgen can impede the ability of progesterone to reduce the GnRH pulse frequency (Eagleson et al., 2000), and is supported by the observation that androgens can suppress hypothalamic progesterone receptor expression (Foecking and Levine, 2005). Interestingly, these results contradict the above-mentioned rat studies, which showed androgen-mediated suppression of GnRH-induced LH release (Foecking and Levine, 2005). This may reflect differences in dose, as rat studies found suppressive effects at high, male-typical doses, but stimulatory effects at low doses. Stimulatory effects on GnRH neurons in female mice were observed with an elevation of DHT that could not fully restore seminal vesicle weight in castrate males, and thus was a “sub-male” level similar to that in hyperandrogenemic women with PCOS. However, in castrate male mice, a male-typical dose of DHT suppressed LH, but was still activating to the GnRH neuron firing rate, suggesting that strong negative feedback at the pituitary potentially masks increased central activity (Pielecka and Moenter, 2006).

Further work has shown how these steroid effects may be mediated transsynaptically and through effects on intrinsic conductances in GnRH neurons. With regard to the former, estrogen receptor-α (ERα), androgen receptor (AR), and progesterone receptor (PR) have consistently not been detected in GnRH neurons, suggesting that afferent neuronal populations transmit steroidal signals to GnRH neurons. A series of experiments examined GABAergic regulation of GnRH neuronal activity following *in vivo* treatment with combinations of estradiol with progesterone, DHT, or both (Sullivan and Moenter, 2005). DHT increased the frequency of GABAergic postsynaptic currents detected in GnRH neurons, as well as current amplitude, whereas progesterone reduced both variables (Figure 5; Sullivan and Moenter, 2005). Combined with the above results on firing properties, these data are consistent with the demonstrated excitatory effect of $GABA_A$ receptor activation on GnRH neurons. The change in frequency of GABAergic postsynaptic currents was not action-potential dependent, in agreement with androgen-dependent changes observed in PNA mice (Sullivan and Moenter, 2004a), providing further evidence that androgens increase the number of GABAergic contacts to GnRH neurons. This suggests that androgens may have activational effects to increase synaptic contacts to GnRH neurons, in addition to possible organizational effects discussed in section 3.2.1. With regard to steroid modulation of intrinsic properties of GnRH neurons, a recent study examined voltage-gated calcium

currents in GnRH neurons exposed *in vivo* to similar steroid milieu (Sun and Moenter, 2010). Voltage-gated calcium channels mediate neuronal influx of calcium, which governs cellular excitability and is critical for hormone release. Progesterone was shown to inhibit calcium currents, whereas DHT activated these currents. These findings are consistent with the inhibitory and activational effects of progesterone and DHT, respectively, on GnRH neuron firing activity. Steroid-sensitive neuromodulatory afferents are likely to mediate these effects, since, as mentioned above, the majority of studies suggest GnRH neurons do not express AR, PR or ERα. This latter point emphasizes the importance of understanding how prenatal androgenization programs changes in neural circuitry.

It is important to note that these studies of steroid regulation of GnRH neuron function were performed in brain slices from animals treated *in vivo* with steroids. The demonstrated results are therefore likely due to genomic effects of steroid activation of nuclear receptors. Steroids can also have rapid, non-genomic effects mediated by membrane receptors, and this was recently demonstrated in GnRH neurons. High physiological levels of estradiol were shown to directly activate GnRH neurons in mice and primates (Abe and Terasawa, 2005; Sun et al., 2010). In mice, this activation depends at least in part on reduction of calcium-activated potassium currents, increases in the sodium-current underlying the slow afterdepolarization, and increases in L- and R-type calcium currents (Sun et al., 2010). The possibility that androgens and progesterone, found at abnormal levels in the circulation in women with PCOS, have similar rapid effects on GnRH neurons remains to be investigated, as does the possibility that PNA alters central steroidogenesis (King et al., 2002).

4.2 Metabolic regulation of GnRH neuron function

Reproductive function is strongly linked to metabolic status. Metabolic abnormalities are extremely common in PCOS and may be causal to reproductive dysfunction in some patients, as suggested by the ability of insulin sensitizers to improve PCOS symptoms (Katsiki et al., 2009). The link between metabolism and reproduction has been particularly well studied with regard to the suppression of fertility by negative energy balance, a major cause of hypothalamic infertility. Numerous studies in this vein have demonstrated that GnRH neurons and/or their afferents can sense and integrate nutritional cues, and that inhibition of GnRH neuronal function is a primary mechanism by which undernutrition suppresses reproductive axis activity (Reame et al., 1984). However, this ability to sense metabolic cues may have vastly different consequences in the context of obesity or insulin resistance, which are frequently manifested in PCOS (Randeva et al., 2012). Further, sex differences in the metabolic control of reproduction suggest that the neural machinery involved in sensing these cues may be regulated by sex steroids, which circulate at abnormal levels in PCOS.

Recent studies have begun to parse out the effects of PCOS-relevant metabolic cues, such as insulin and glucose, on GnRH neuron function. The role of insulin was examined using a novel conditional knockout model in which the insulin receptor was genetically deleted from GnRH neurons (GnRH-IRKO) (Divall et al., 2010). Perhaps surprisingly, GnRH-IRKO mice exhibit normal timing of puberty, estrous cycle length, fertility, and LH levels. This is in marked contrast to neuronal insulin receptor knockout (NIRKO) mice, in which the

insulin receptor is deleted throughout the brain; these mice display subfertility and reduced LH hypothesized to result from impaired GnRH secretion (Brüning et al., 2000). NIRKO mice also exhibit obesity and other phenotypes that may cause secondary hypothalamic infertility. Alternatively, insulin may be important in regulating GnRH neuronal function through transsynaptic mechanisms, similar to other metabolic cues (Sullivan and Moenter, 2004b). Given that much of the steroid regulation of GnRH neurons is also transsynaptic (Sullivan and Moenter, 2005), metabolic and steroid feedback cues may be integrated upstream. The role of insulin receptors directly on GnRH neurons remains unknown, but they may become important under pathological conditions such as nutritional challenge, and thus remain of interest with respect to PCOS.

There is abundant evidence for circulating glucose as a primary cue regulating the interaction between energy and fertility (Wade et al., 1996). The LH pulse frequency is reduced by insulin-induced hypoglycemia or by glucose anti-metabolites (Bucholtz et al., 1996; He et al., 1999; Ohkura et al., 2004; Rodríguez et al., 1999), indicating that short-term changes in blood glucose regulate GnRH neuronal activity. The brain interstitial glucose level is ~30% of the blood concentration. Thus, in diabetic and pre-diabetic PCOS patients, abnormal elevations in blood glucose may activate GnRH neurons. Only recently has this been examined directly at the level of the GnRH neuron. The first examination of glucosensing by GnRH neurons demonstrated that over half of GnRH neurons are responsive to a change in glucose concentration within the physiological range (Zhang et al., 2007). As glucose concentrations decline, the GnRH neuronal firing rate also declines. These recordings were made in a high Mg^{2+}/low Ca^{2+} extracellular solution designed to minimize presynaptic neurotransmitter release, suggesting that these effects are mediated directly at the GnRH neuron. 60% of GnRH neurons were shown by single-cell RT-PCR to express mRNA for Kir6.2, a potassium channel implicated in glucosensing in other cell types, including pancreatic beta cells. Although these findings suggested that glucosensing was mediated by K_{ATP} current, this was not directly demonstrated. A subsequent *in vivo* study examined the role of this K_{ATP} channel in the suppression of LH secretion by fasting (Huang et al., 2008). Fed and fasted mice received intracerebroventricular injections of the K_{ATP} antagonist tolbutamide, but both groups responded showed similar LH responses to the drug, indicating that K_{ATP} current was not increased in fasted animals. This study also showed that sulfonylurea-1 null mice, which lack intact K_{ATP} channels, exhibit a normal suppression of LH in response to fasting. Since glucose appears to be the primary mediator of reproductive channels are not axis suppression by fasting (Wade et al., 1996), these results indicate that K_{ATP} a major component of glucosensing in this system.

A subsequent study confirmed and extended these findings and postulated a novel glucosensing mechanism (Roland and Moenter, 2011b). Of particular relevance to PCOS, this study also examined the influence of steroids on glucosensing by using female mice that were ovariectomized and treated *in vivo* with capsules containing oil, DHT, estradiol, or DHT+estradiol. As previously established, GnRH neurons showed reductions in firing activity in response to reduced extracellular glucose (Figure 6; Roland and Moenter, 2011b). Importantly, these responses were observed at glucose concentrations within the physiological range for cerebrospinal fluid (0.2 to 4.5 mM). *In vivo* androgen treatment

diminished sensitivity to changes in extracellular glucose; however, co-treatment with estradiol mitigated this effect, and glucosensing remained intact in cells from animals treated with both DHT and estradiol. This observation suggests that glucosensing would remain functional in the context of the PCOS endocrine milieu. Consistent with this prediction, GnRH neurons from PNA mice showed similar responses to glucose changes as control mice. Mechanistically, the effects of glucose on neuronal activity were mediated by AMP-activated protein kinase (AMPK), a cellular energy sensor. The AMPK activator AICAR inhibited GnRH neuron firing (Figure 6), and both AICAR and low glucose activated an as-yet unidentified nonspecific cation current. These findings were particularly interesting from the standpoint of AMPK as a novel regulator of GnRH neuron function, and as the mechanistic target of a common PCOS therapeutic, metformin. Further, AMPK lies downstream of numerous other metabolic signals, such as leptin, ghrelin, and adiponectin (Kola et al., 2006), suggesting that GnRH neurons may also be susceptible to regulation by these metabolic cues. We propose that in women with PCOS and poor blood glucose control, abnormally high blood glucose levels may exacerbate elevated GnRH neuron activity, perpetuating increased GnRH release. One caveat to these experiments is that acute glucose changes were studied, but it is unclear how responses would differ under conditions of chronically elevated glucose and/or insulin.

Obesity is a complex condition marked by altered levels of insulin, glucose, and adipokines such as leptin, among many other factors. Obesity is prevalent in PCOS (Lim et al., 2012), raising the question of how this drastically altered metabolic state affects reproductive neuroendocrine function. In obese women, LH levels are generally reduced (Grenman et al., 1986). This appears to be due to a reduction in LH pulse amplitude (Jain et al., 2007), although it should be pointed out that lower amplitude pulses are more difficult to detect, and thus frequency could also be different. This observation holds true for obese women with PCOS (Morales et al., 1996; Taylor et al., 1997). At the pituitary, LH responsiveness to GnRH is inversely related to body mass index (Pagán et al., 2006), suggesting that effects of obesity may be mediated at this level; however, a contribution of changes in GnRH pulse amplitude cannot be ruled out. Given the demonstrated influence of glucose and other metabolic cues such as leptin on GnRH neurons (Roland and Moenter, 2011c; Sullivan et al., 2003, 2002), obesity is likely to alter GnRH neuron output. The importance of these potential changes relative to the established pituitary effects of obesity is unknown.

The observation of reduced LH levels in obesity suggests that adiposity is not a major determinant of neuroendocrine dysfunction in PCOS. However, the ability of obesity to induce a PCOS-like syndrome was recently demonstrated in a diet-induced obesity mouse model (Brothers et al., 2010). Whereas obese DBA/2J mice show reduced LH levels and reproductive phenotypes consistent with hypogonadotropic hypogonadism (Tortoriello et al., 2004), similar to obese women, this particular mouse strain (CD1/129SvJ/C57Bl6) developed a PCOS-like syndrome under conditions of high-fat feeding, with no other experimental manipulations. High-fat-fed mice had elevated LH and testosterone levels, irregular estrous cycles with a predominance of diestrus, and reduced numbers of corpora lutea, similar to PNA mice. Isolated pituitaries from these mice had increased LH secretion in response to exogenous GnRH, and increased GnRH receptor mRNA expression, which may be caused directly by hyperinsulinemia. High-fat diet-induced reproductive defects

were partially reversed by targeted genetic deletion of the insulin receptor from the pituitary (pitIRKO). High-fat-fed pitIRKO mice had normal LH levels, were fertile, and had numbers of corpora lutea intermediate between that of high-fat-fed wild-type and lean mice. Testosterone levels in pitIRKO mice were not reported. pitIRKO mice had reduced pituitary GnRH receptor expression and reduced LH response to GnRH injection relative to normal-weight controls, suggesting that circulating insulin acts on pituitary insulin receptors to increase GnRH receptor expression even in the lean state. Additional experiments showed that under conditions of profound whole-body insulin resistance in the obese state, the pituitary remains insulin sensitive, in contrast to many other peripheral tissues, leading to excessive insulin-driven LH production. These findings suggest that diet-induced obesity combined with a sensitive genetic background can cause a PCOS-like syndrome driven in part by pituitary dysfunction. However, as fertility and corpora lutea numbers were incompletely rescued by pituitary insulin receptor deletion, obesity may have effects at other levels of the reproductive axis. In this regard, the ovary also remains insulin-sensitive in the obese state, and deletion of insulin receptors from ovarian theca interstitial cells also partially rescues obesity-induced infertility (Wu et al., 2013, 2012). The effect of obesity on GnRH neuronal function remains to be investigated. The difference in reproductive outcomes between several high-fat diet models is intriguing (Brothers et al., 2010; Tortoriello et al., 2004), and it would be interesting to compare levels of adiposity between these strains. Several metabolic factors circulate at levels proportional to adiposity, and in severe obesity, systems lose their ability to compensate for these changes, such as in type 2 diabetes. Thus, varying degrees of obesity may differentially affect GnRH pulsatility.

Conclusions and future directions

Disruptions in the pattern of GnRH release contribute to reproductive dysfunction in many women with PCOS. Changes in GnRH pulsatility may be in part programmed by fetal androgen excess, which may permanently alter steroid negative and positive feedback regulatory mechanisms via changes in glial contacts and synaptic inputs to GnRH neurons, neural steroid receptor expression and distribution, and neuromodulator expression (Table 1). Fetal androgen excess may also produce indirect effects mediated by ovarian and metabolic programming. However, even in the absence of a primary neural defect, changes in steroid feedback and altered metabolic cues, secondary to other PCOS abnormalities, can have activational effects to disrupt normal GnRH neuron function (Figure 7). Treatments that address these aberrant interactions and/or target the GnRH system may facilitate the restoration of fertility.

Work in PNA models has begun to identify early antecedents of adult reproductive neuroendocrine and metabolic dysfunction and has demonstrated the emergence of numerous abnormalities in androgenized animals well before puberty. These studies should be extended to the mouse model to obtain further insight on possible early changes in GnRH neurocircuitry and function following prenatal androgenization, as well as the cellular and molecular mechanisms involved in these changes. It is becoming clear that developmental androgen excess affects a multitude of systems from very early on, suggesting that the development of some phenotypes in PNA animals may be indirectly programmed by the perturbation of metabolic and other factors. Studies in adult animals have shown

unequivocally that the reproductive neuroendocrine system is highly sensitive to metabolic input; however, it is unknown at what point in development this sensitivity first emerges, and whether early metabolic changes can influence the ontogeny of the GnRH system. Genetic tools in mice, such as conditional inducible knockout models, may be useful in this regard to interrupt receptor expression at different points in the development of specific tissues. The continued identification of specific factors, metabolic and other, mediating the effects of prenatal androgen excess is essential for identifying possible early interventions, which these recent studies (Abbott et al., 2010) suggest could be dietary/behavioral in addition to pharmacological. The early emergence of PCOS symptomology in PNA animals parallels clinical observations, such as elevated steroid levels at birth in female offspring of women with PCOS (Barry et al., 2010), and pubertal reductions in hypothalamic steroid sensitivity in obese hyperandrogenemic girls (Beller et al., 2012). Screening and intervention at key postnatal time points may alter the developmental trajectory of the reproductive neuroendocrine system, thereby preventing or diminishing the severity of PCOS in adulthood. Continued use of the PNA models will be invaluable for testing these potential interventions.

Future work should also continue to address the neurobiological, cellular, and molecular mechanisms underlying steroidal and metabolic dysregulation of GnRH neurons in adulthood. Much is known about the long-term effects of steroids to regulate GnRH release through nuclear receptor signaling, but it has recently come to light that steroids can also have rapid effects on GnRH neuronal activity mediated by membrane receptors (Kenealy and Terasawa, 2011; Moenter and Chu, 2012). This should be investigated with respect to the PCOS steroidal milieu; rapid effects of androgens remain uninvestigated. Kisspeptin has also emerged as an important regulator of GnRH neuronal function (Goodman and Lehman, 2012), and plasma kisspeptin is elevated in women with PCOS (Jeon et al., 2013; Panidis et al., 2006), but PCOS models have not explored this area in depth (Witchel and Tena-Sempere, 2013). Key sex differences in the kisspeptin system suggest that it may be susceptible to developmental or activational effects of steroids (Kauffman, 2010; Smith, 2013). Other neuromodulators such as neurokinin B and the inhibitory factor gonadotropin-inhibiting hormone (GnIH) may be important. As we continue to elucidate the fundamental mechanisms of cellular regulation of GnRH neurons, such as autoregulation by GnRH (Chen and Moenter, 2009), these findings should be applied to prenatal and adult treatment PCOS models. The novel use of fast-scan cyclic voltammetry to directly measure GnRH concentrations in the brain slice has substantiated electrophysiological studies with measures of hormone release, adding further information regarding the frequency and amplitude of GnRH release in the median eminence in response to experimental treatments (Glanowska et al., 2012). This will be a valuable tool for future studies. Finally, future work should also continue to take advantage of the genetic manipulability of mice, for example, by generating mice expressing the recent PCOS-linked gene polymorphism to determine its function. Elucidation of the mechanisms underlying dysregulation of GnRH neurons in adulthood may yield novel targets for therapeutic development.

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Highlights

- Disrupted reproductive neuroendocrine function is a frequent finding in PCOS.
- Changes in GnRH neuron function can result from prenatal androgen excess in females.
- Androgens and glucose can activate GnRH neurons in adult females.

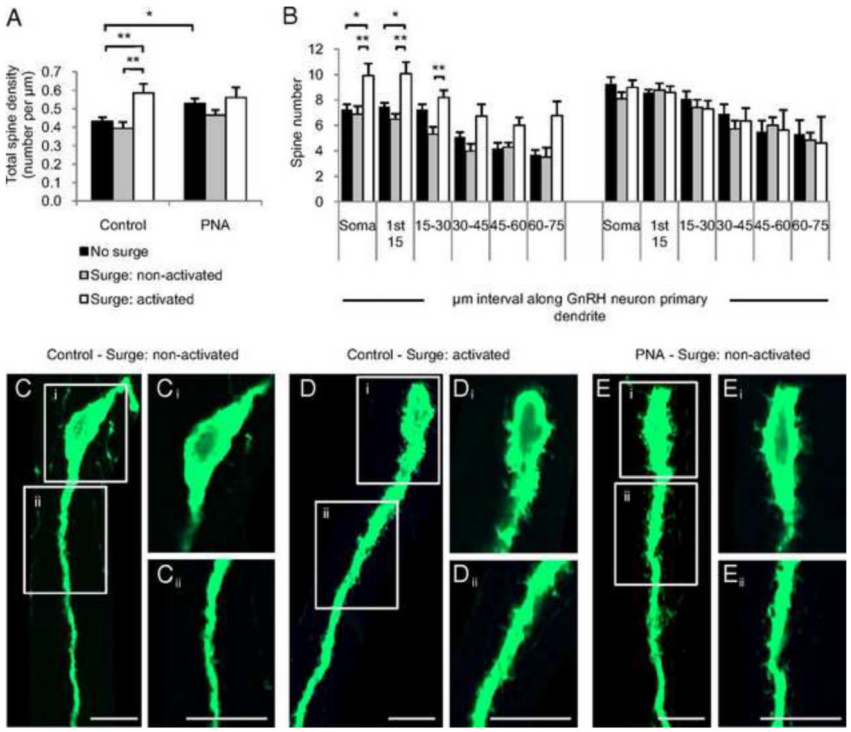

Figure 1.

PNA mice fail to show an increase in dendritic spines in c-fos-expressing GnRH neurons at the time of the estradiol benzoate (EB)-induced surge. A, GnRH neuron spine density is increased in control mice in c-fos-positive neurons at the time of the surge, compared to inactivated neurons and neurons in untreated mice. PNA mice do not show this increase. Untreated PNA mice exhibit a greater spine density than untreated controls. B, In c-fos-expressing GnRH neurons in EB-treated control animals, the increase in spines is detected at the soma and first 30 um of the primary dendrite. C-E, Projected confocal images of representative GnRH neurons. i-ii, Individual confocal images (450 nm optical thickness) from selected subregions of panels C–E. *, P<0.05. From (Moore et al., 2013) with permission.

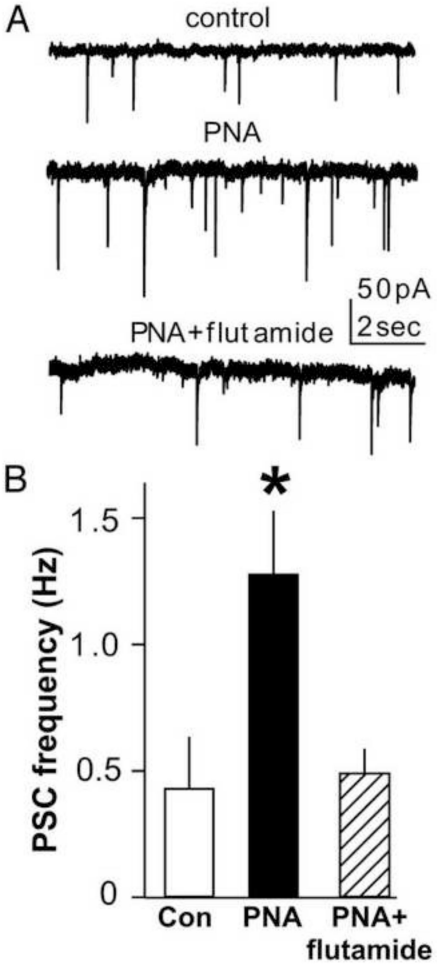

Figure 2.

PNA mice exhibited increased frequency and amplitude of GABAergic postsynaptic currents in GnRH neurons that is reversed by treatment with flutamide *in vivo*. A, Representative current traces from a PNA, control, and PNA mouse treated with flutamide. B, Summary bar graph indicating the increase in mean GABA PSC frequency in PNA mice and reversal by flutamide. *, $P<0.05$. From (Sullivan and Moenter, 2004a) with permission.

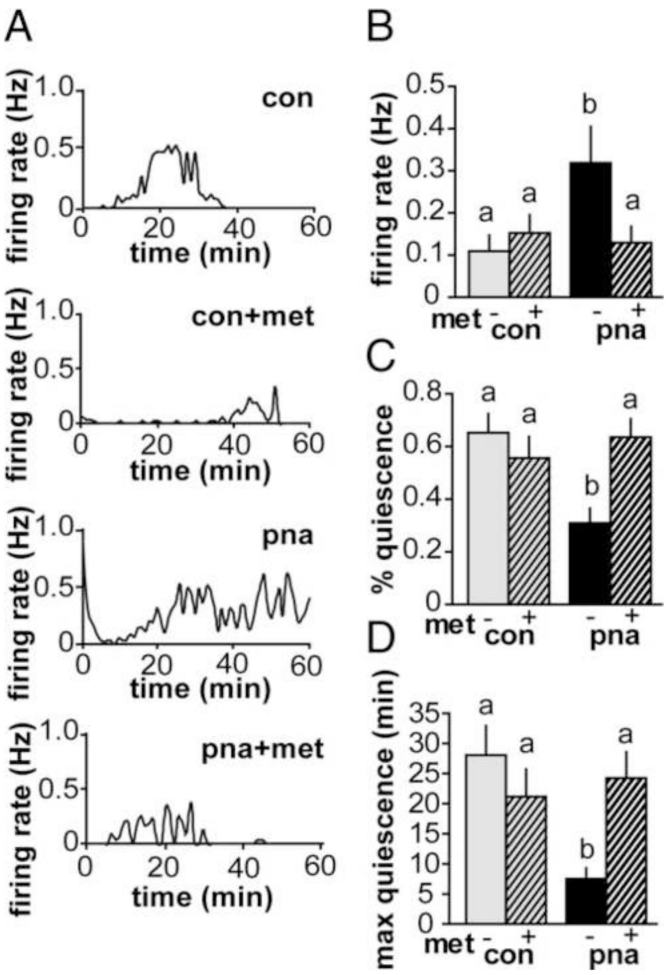

Figure 3.

PNA mice exhibit increased activity of GnRH neurons that is reversible by treatment with metformin *in vivo*. A, Representative plots of firing rate over time from control (con), control+metformin (con+met), PNA, and PNA+metformin (PNA+met) treatment groups illustrating elevated GnRH neuronal activity in untreated PNA animals. B, Bar graphs summarizing the effects of PNA and metformin on measures of firing rate, percent of time cells were quiescent (1 event/min), and maximum duration of quiescence during a one-hour recording. Different letters indicate significant differences among groups. From (Roland and Moenter, 2011a) with permission.

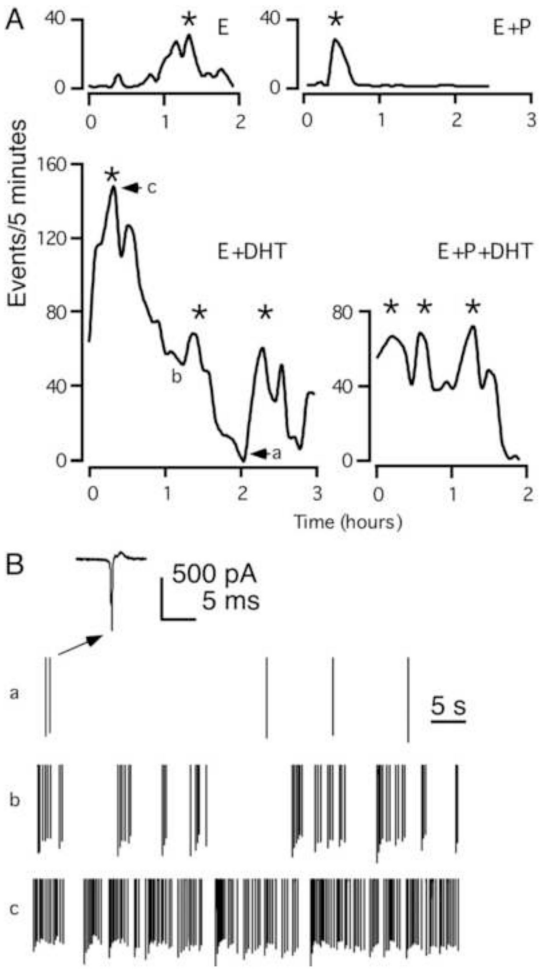

Figure 4.

Androgen increases GnRH neuron firing activity in females. A, Representative plot of firing rate over time in GnRH neurons from mice treated with estradiol (E) alone, E+progesterone (P), E+DHT, or E+P+DHT. Asterisks mark increased firing rate determined by the Cluster pulse-detection algorithm (Veldhuis and Johnson, 1986). Lowercase letters in the E+DHT example indicate areas detailed in B. B, An individual action current is shown on top. a, b, and c are 1-minute excerpts of action currents indicated in A. These illustrate action currents during low (a), medium (b), and high (c) firing rates in this cell. From (Pielecka et al., 2006) with permission.

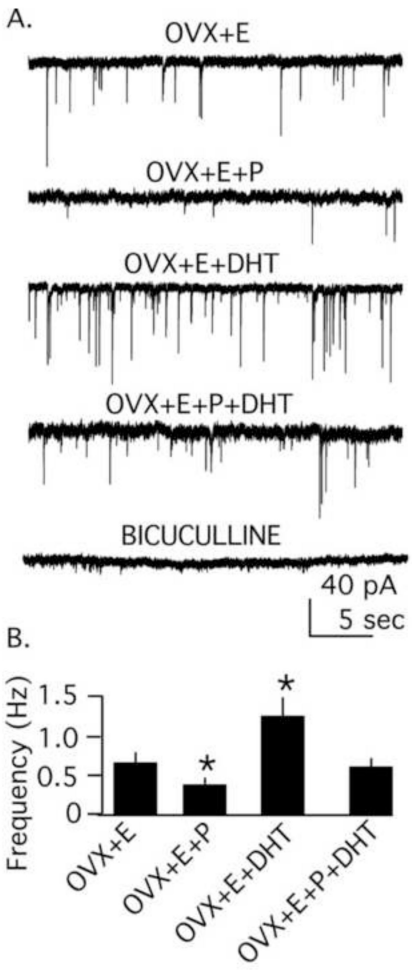

Figure 5.

DHT increases and P reduces GABAergic PSC frequency in GnRH neurons from female mice. A, Representative current traces showing GABAergic PSCs in GnRH neurons from OVX mice treated with estradiol (E) alone, E+progesterone (P), E+DHT, or E+P+DHT. PSCs are blocked by the $GABA_A$ receptor antagonist bicuculline. B, Summary bar graph. *, $P<0.05$ versus OVX+E. From (Sullivan and Moenter, 2005) with permission.

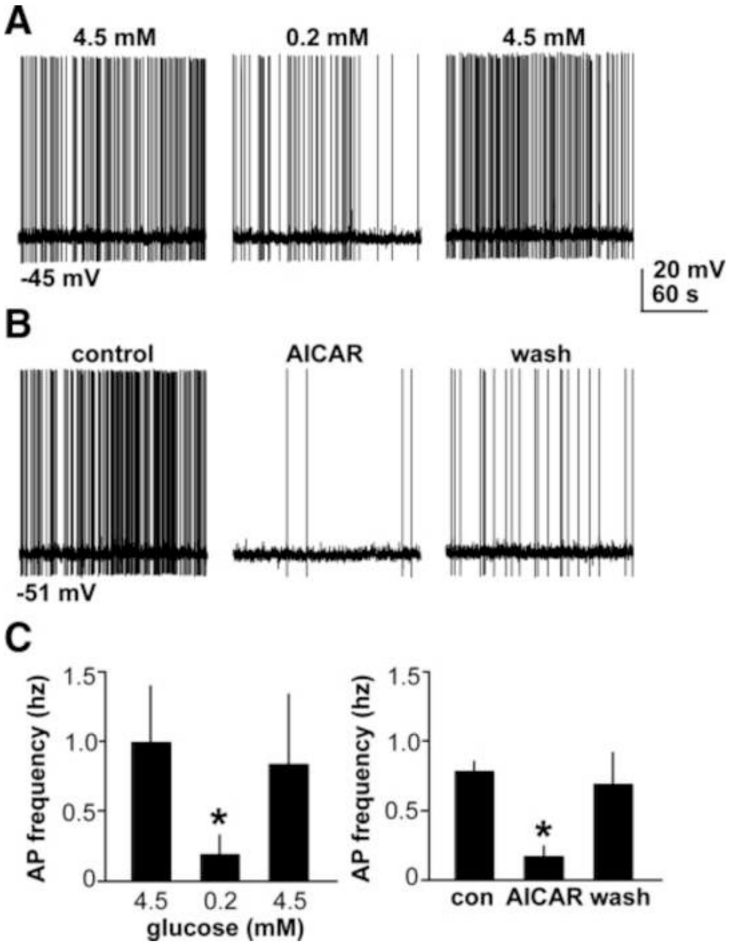

Figure 6.

Low glucose and an AMPK activator inhibit GnRH neuron firing activity. A, Representative current-clamp traces from a GnRH neuron in response to a switch in extracellular glucose from 4.5 mM to 0.2 mM. Low glucose suppresses firing activity. B, Representative current-clamp traces from a GnRH neuron in response to acute application of the AMPK activator AICAR. AICAR has a similar inhibitory effect as low glucose. C, Mean action potential (AP) frequency before and after low glucose or AICAR, and after 15–25 min of washout (wash). *, $P < 0.05$. s, second; con, control. From (Roland and Moenter, 2011b) with permission.

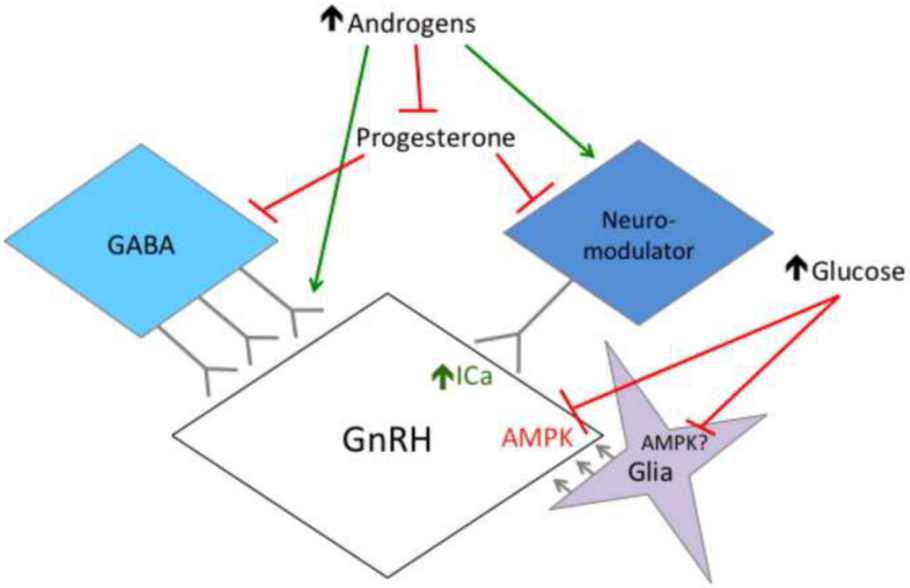

Figure 7.

Summary of activating effects of androgens and glucose on GnRH neurons. Androgens activate (green lines) GnRH neurons by increasing excitatory GABAergic neurotransmission, enhancing neuromodulator-evoked calcium currents, and blocking (red lines) the inhibitory effects of progesterone on these parameters. Glucose activates GnRH neurons by suppressing the inhibitory influence of AMPK, which may be expressed in the GnRH neuron or in glial cells.

Table 1

Summary of neuroendocrine defects in PNA models and associated mechanistic findings.

Species	Prenatal steroid	↑ LH levels	↑ LH pulse frequency	E +FB defect	E −FB defect	P −FB defect	Associated findings
Rhesus monkey	T (GD 40-80)	Yes[a,b]	unknown	No[c]	Yes[c]	Yes[d]	
Sheep	T (GD 30-90)	Yes[e,f,g]	Yes[h,i]	Yes[f,j,k]	Yes[g]	Yes[l,m]	Absence of c-Fos expression in GnRH neurons following OVX+E surge paradigm[n] Reduced synaptic contacts to GnRH neurons, reduced glial ensheathment (OVX+E[o] or intact+prostaglandin f2α injection[p]) Reduced cells expressing dynorphin, neurokinin B, PR in arcuate nucleus; loss of neuropeptide coexpression in kisspeptin cells (OVX+standard hormone treatment)[q]
Rat	T (GD 16-19)	Yes (3 mg dose)[r]	Yes (1 mg dose)[s]	Yes[s]	unknown	unknown	Reduced PR expression in MBH and POA on proestrus (OVX+Er or intact[s]) Impaired estradiol-induced increase in PR expression (OVX)[s]
Mouse	DHT (GD 16-19)	Yes[t,u]	unknown	No[v]	Yes[v]	unknown	Elevated GnRH neuronal activity (intact)[u] Increase in GABAergic neurotransmission/GABA release sites on GnRH neurons (intact)[t] Increased spine density on GnRH neurons in rPOA; failure of E-induced surge to increase spines (OVX+E)[v]

[a] Abbott et al., 2005

[b] Dumesic et al., 1997

[c] Steiner et al., 1976

[d] Levine et al., 2005

[e] Manikkam et al., 2008

[f] Sharma et al., 2002

[g] Sarma et al., 2005

[h] Savabieasfahani et al., 2005

[i] Steckler et al., 2009

[j] Herbosa et al., 1996

[k] Unsworth et al., 2005

[l] Robinson et al., 1999

[m] Veiga-Lopez et al., 2008

[n] Wood et al., 1996

[o] Kim et al., 1999

[p] Jansen et al., 2011

[q] Cheng et al., 2010

[r] Wu et al., 2010

[s] Foecking et al., 2005

[t] Sullivan and Moenter, 2004a

[u] Roland and Moenter, 2011a

[v] Moore et al., 2013

LH, luteinizing hormone; E, estradiol; FB, feedback; T, testosterone; OVX, ovariectomized; PR, progesterone receptor; GD, gestational day; MBH, mediobasal hypothalamus; DHT, dihydrotestosterone; GnRH, gonadotropin releasing hormone; GABA, gamma aminobutyric acid; rPOA, rostral preoptic area