Prepubertal Gonadotropin-Releasing Hormone Neuron Activity and the Effects of Prenatal Androgen Exposure

by

Eden A. Dulka

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Doctoral Committee:

Professor Suzanne M. Moenter, Chair
Associate Professor Catherine Collins
Professor Carol F. Elias
Professor David P. Olson
Associate Professor Michael A. Sutton

While on this ride called "life”,

you have to take the good with the bad, smile when you're sad,

love what you've got and remember what you had.

Always forgive, but never forget.

Learn from your mistakes, but never regret.

People change. Things go wrong.

Just remember, the ride goes on.

-unknown

Eden A. Dulka

edulka@umich.edu

ORCID iD: 0000-0002-7724-8923

Dedication

To my little brother A.J.,

I'd rather be given one more day with you then authorship on 10,000 Nature papers

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List of Abbreviations

ACSF	artificial cerebrospinal fluid
AMH	anti-Müllerian hormone
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AVPV	anteroventral periventricular
BMI	body mass index
CL	corpus luteum
CNO	clozapine-N-oxide
CV	coefficient of variation
E	Estradiol
DHT	5α-dihydrotestosterone
DMSO	Dimethyl sulfoxide
DREADDs	Designer Receptor Exclusively Activated by Designer Drugs
FSH	follicle-stimulating hormone
FWHM	full-width half-maximum
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
GWAS	genome-wide association studies
HA	Human influenza hemagglutinin

hM3Dq	human M3 muscarinic receptor
hM4Di	human M4 muscarinic receptor
HPG	hypothalamic-pituitary-gonadal
Hz	hertz
IP	intraperitoneal
ISI	interspike interval
KS	Kolmogorov-Smirnov
LH	luteinizing hormone
LSD	least significant difference
mPSCs	miniature postsynaptic currents
NIH	national institutes of health
OVX	ovariectomized
P	Progesterone
PCOS	polycystic ovary syndrome
PNA	prenatally androgenized
QC	quality control
SC	subcutaneous
SEM	standard error of the mean
sPSCs	Spontaneous postsynaptic currents
VO	vaginal opening

Abstract

Reproductive function is governed by the hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) neurons are central mediators of the reproductive axis and release GnRH, which controls pituitary and gonadal function. Polycystic-ovary syndrome (PCOS) is the leading cause of infertility in reproductive-aged women. PCOS is characterized by an upregulation in luteinizing hormone (LH), and thus GnRH release. Endocrine abnormalities of PCOS, including increased androgens, high-frequency LH pulses, and disrupted reproductive cycles, are present in prenatally androgenized (PNA) animal models. GnRH neuron activity in adult female PNA mice is also elevated. Clinical studies in girls with hyperandrogenemia revealed disrupted LH pulsatility prior to adulthood, suggesting that aspects PCOS may manifest before puberty.

In this dissertation we demonstrate that GnRH neurons are prepubertally active and that this activity is disrupted in PNA mice. To examine this activity we used electrophysiological extracellular recordings conducted from GFP-identified GnRH neurons in brain slices from control and PNA mice at postnatal weeks 1-4. We found that GnRH neuron activity was present in both males and females at all time points tested. In control female mice prepubertal activity of GnRH neurons increased during early life, with firing being highest at 3 weeks. In contrast, firing activity in PNA mice did not increase across the postnatal period. To investigate if the downstream reproductive axis plays a role in GnRH neuron firing rate of PNA mice, we tested hypotheses that 1)

increased GnRH neuron firing in adult PNA mice is due to ovarian factors, specifically androgens, and 2) that gonadal factors have minimal influence on GnRH neuron firing in 3-week old mice. Interestingly, removing ovarian inputs in adult PNA mice decreased firing rate and addition of androgen implants in ovariectomized mice restored firing to that of PNA sham-operated mice, suggesting that ovarian androgens are a primary contributor driving increased GnRH neuron firing in adult PNA mice. In 3-week old control and PNA mice however no differences were found between sham-operated and ovariectomized groups, suggesting a minimal role for ovarian factors on GnRH neuron firing in 3-week old PNA mice. Because ovarian inputs did not affect GnRH activity in 3-week old PNA mice, we next hypothesized that prepubertal GnRH neuron activity plays a role in establishing adult reproductive function and in setting up adult activity of the GnRH neuronal network. Either activating (hM3Dq) or inhibitory (hM4Di) designer receptors exclusively activated by designer drugs (DREADDs) were targeted to GnRH neurons to allow for remote control of neuronal activity. Injection of clozapine n-oxide (CNO) produced the expected changes in luteinizing hormone (LH) release from the pituitary in control studies. CNO was administered between two and three weeks of age, the period when GnRH neuron activity was reduced in PNA mice. Reducing GnRH neuron activity during development decreased adult GnRH neuron firing rate. Interestingly, neither manipulation altered the timing of pubertal makers. Thus GnRH neuron activity programs changes in the adult neuronal network governing reproductive function but altering prepubertal GnRH neuron activity cannot fully explain the phenotypes found in PNA mice. Overall this body of work indicates that the reproduce axis is active prior to puberty. These results also support a model in which prepubertal

alteration of GnRH neuronal activity programs changes in the adult GnRH neuron network and indicate that androgens play a role in the manifestation of reproductive abnormalities observed in PNA mice.

Chapter 1 Introduction

The primary goal of this chapter is to review the knowledge in the field at the time this dissertation project was developed.

Significance

Reproductive function is required for propagation of genetic offspring and species survival. It encompasses a variety of physiological processes including gametogenesis, fertilization, pregnancy maintenance, lactation, pubertal processes and reproductive senescence. Disruptions in these physiological processes therefore can result in infertility, a problem that is estimated to affect 1 in 6 couples (1). In many of these cases, the cause behind infertility remains unexplained. The reproductive system also plays a major role in driving sex steroid production. These steroids not only have action on reproductive tissues but also act on other systems such as the muscular and skeletal systems. Because outputs of the reproductive axis can affect multiple areas of physiology, compromised reproductive function can lead to a disruption in overall health. Advancing our basic understanding of reproductive function in both normal and disease states can therefore shed light on our basic understanding of overall physiology and aid those with reproductive complications.

Reproductive Axis Overview

Reproductive function is maintained through the hypothalamo-pituitary-gonadal (HPG) axis (Figure 1-1). Gonadotropin-releasing hormone (GnRH) neurons, located in the preoptic area and hypothalamus, act as central integrators relaying environmental cues to downstream components of the reproductive system and responding to physiological feedback. Release of GnRH stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion from the anterior pituitary. Both LH and FSH travel though the circulation to act on gonads and aid in gametogenesis and sex steroid production. Sex steroids then feed back to the upstream components of the reproductive axis. Because GnRH neurons have been shown to lack most steroid receptors, steroid feedback is primarily at neurons upstream of GnRH neurons, which subsequently convey these feedback signals to GnRH neurons thus regulating their activity and release. In females, fluctuations in feedback control of GnRH release frequency are required for follicular maturation and successful ovulation. When this feedback system is disrupted it can lead to infertility disorders such as polycystic ovary syndrome (PCOS) in which both steroid feedback and LH/GnRH release are altered.

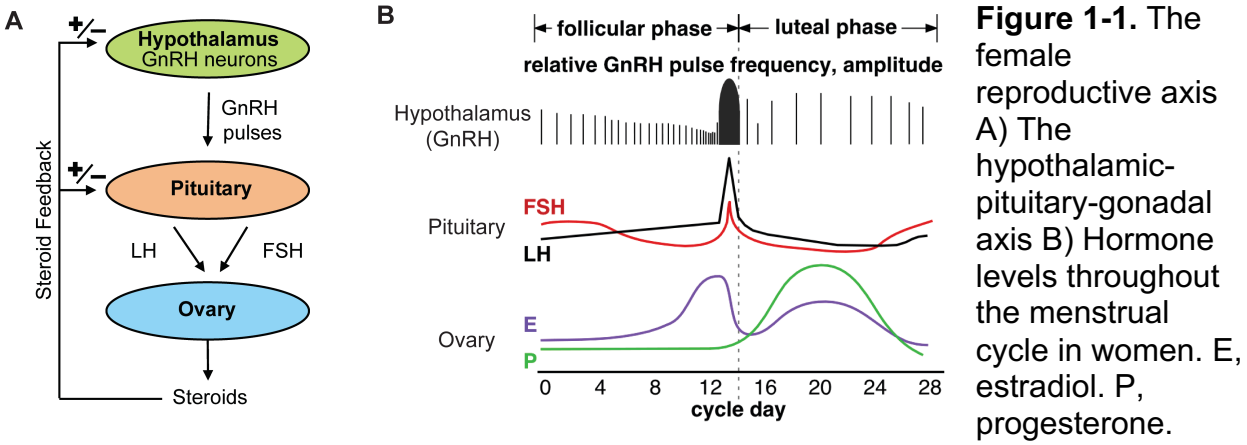

Figure 1-1. The female reproductive axis A) The hypothalamic-pituitary-gonadal axis B) Hormone levels throughout the menstrual cycle in women. E, estradiol. P, progesterone.

The GnRH "pulse generator"

Early studies in monkeys demonstrated a tonic mode of LH secretion across most of the female cycle with the exception of a single LH surge (2), which precedes ovulation. One pitfall of these studies was the low sampling frequency did not allow visualization of a then recently demonstrated patterned release of LH. A seminal study in ovariectomized monkeys, in which sampling frequency was increased from daily to as often as 10-min intervals, demonstrated a beautiful pattern of oscillatory LH release (3). LH pulses have a sharp peak and then subsequent decay period prior to the next peak occurring. Because no evidence for a short feedback loop via LH itself could be identified to account for these LH oscillations (4), and GnRH had recently been sequenced (5,6), scientists began to investigate if a neuronal component may be responsible for oscillatory LH output. Evidence showing a strong association between oscillations of multiunit activity recorded on electrode arrays placed in the hypothalamus and LH release (7) supported this idea. Subsequent studies in the sheep showed a strong correlation between GnRH and LH pulses through portal blood measurements (8). These studies supported that GnRH release occurs in a pulsatile fashion, with a sharp rise and subsequent decrease separated by basal levels of essentially no hormone release.

The pulsatile nature of GnRH release is crucial for reproductive function, particularly in driving changes in gonadotropin levels necessary for a successful reproductive cycle in females (Figure 1-2). Studies in which endogenous GnRH pulses were ablated found that administration of a continuous GnRH treatment markedly suppressed LH and FSH release(9). Furthermore, varying GnRH administration

frequency demonstrated that increasing GnRH frequency favors LH release whereas lower frequency favors FSH release(10). In most instances, GnRH and LH pulses are well correlated and LH pulses can be used as a bioassay for GnRH release. Exceptions include when pulse frequency is very high, for example, in thyroidectomized ewes the frequency of GnRH release (about 4-5 pulses per hour) is too fast to be mirrored by LH pulses (11). Another example is during the preovulatory LH surge, when GnRH release is not pulsatile and the GnRH surge lasts longer than the correlated LH secretion (12,13).

GnRH pulse frequency varies throughout the cycle leading to the regulation of LH and FSH. Changes in pulse frequency are governed by steroid feedback. During most of the cycle, GnRH pulses are suppressed via negative feedback of estradiol and progesterone. Studies in ovariectomized sheep demonstrated that progesterone and estradiol diminish both GnRH and LH pulsatile secretion (14). It was also shown that estradiol decreases the amplitude of elicited LH pulses (via administration of GnRH) supporting estradiol has an effect on the pituitary as well as the brain(15). Negative feedback of estradiol decreases GnRH pulse amplitude (16), but rising estradiol also increases GnRH/LH pulse frequency (13,17). Because progesterone receptor expression has been shown to be estrogen dependent (18), estradiol is also needed for effects of progesterone to decrease GnRH pulse frequency. At the beginning of the follicular phase, GnRH pulse frequency is low due to progesterone presence during the previous luteal phase. Low GnRH pulse frequency allows a rise in FSH, which stimulates development of ovarian follicles. As a dominant follicle is recruited, it secretes inhibin, which lowers FSH. Concurrently, GnRH pulse frequency increases

allowing for LH to increase in the late follicular phase. This increase in LH leads to a sustained increase in estradiol and estradiol feedback switches from negative to positive, inducing a GnRH surge. Positive feedback via estradiol to increase LH pulse frequency is independent of progesterone (17). Following the ovulatory surge, progesterone is secreted from a group of remnant cells of a ruptured follicle known as the corpus luteum (CL). This increase in progesterone decreases GnRH pulse frequency and the switch from positive to negative feedback of estradiol once again occurs. This progesterone feedback is removed as degeneration of the corpus luteum takes place, and if no pregnancy is achieved, this allows for the follicular phase to start again. In patients with some infertility disorders, such as PCOS, these typical fluctuations in GnRH pulse frequency important for driving the cycle do not occur.

Polycystic Ovary Syndrome (PCOS)

PCOS is the leading cause of infertility and reproductive complications in female patients of reproductive age. PCOS is a multifaceted disorder diagnosed based on criteria that include the presence of hyperandrogenemia, oligoovulation or anovulation, and/or polycystic ovarian morphology. The “Rotterdam” criteria are most commonly used to diagnose PCOS patients and state that patients must present with a minimum of two of the three criteria. It is estimated that up to 20% of women are affected by PCOS under these criteria (19). Regardless of the criteria used for diagnosis, PCOS patients are more likely to suffer from additional health defects such as obesity, insulin resistance, cardiovascular disease, and mental health disturbances (20). Due to its complexity, the etiology and mechanisms underpinning PCOS are not fully understood.

PCOS patients present with an array of physiological disruptions of the reproductive, metabolic and endocrine systems. Many patients with PCOS suffer from menstrual irregularities including anovulation. These irregularities are thought to be due to disrupted follicular growth potentially driven by excess androgens. Hyperandrogenemia is major contributor in PCOS affecting up to 60% of PCOS patients that meet the Rotterdam criteria (21), and all women that meet the classic NIH or Androgen Excess Society criteria (22,23). Although the ovary is the site of this increased androgen synthesis, both mechanisms within the ovary and increased upstream endocrine signals likely underlie increased androgen production. With regard to the latter, in women with PCOS both LH pulse frequency and amplitude are upregulated (24). These changes are thought to be a result of increased GnRH pulse frequency and increased pituitary response to GnRH (25), respectively. The upregulation of the GnRH pulse generator in women with PCOS may be due to impaired progesterone feedback mechanisms. Studies in PCOS women suggest that higher levels of progesterone are required to reduce LH pulse frequency than in control patients (26). Androgens are thought to play a role in this impaired progesterone feedback. Studies using the antiandrogenic drug flutamide demonstrated it restored sensitivity to progesterone/estradiol negative feedback(27). This indicates central feedback control of GnRH is affected in an androgen-dependent manner. Interestingly, flutamide alone had no effect on LH pulses but did reduce LH pulse frequency in the presence of progesterone and estradiol (27).

Subsequent studies in animal models further investigated possible mechanisms underlying these clinical observations that androgens may interfere with progesterone

inhibition of the GnRH network. Ovariectomized mice were given combinations of implants containing estradiol, progesterone, and the nonaromatizable androgen 5α-dihydrotestosterone (DHT) for 8-12 days. Of note, the DHT implants were designed to mirror levels of increased androgens observed in patients from the above PCOS studies, that is they were designed to sustain a higher-than-normal female, yet sub-male, dose of androgens. Progesterone in the presence of estradiol suppressed both excitatory GABAergic input to GnRH neurons (28) and GnRH firing (29). When DHT implants were also given, the progesterone-induced suppression was compromised for both GABAergic input (28) and GnRH neuron firing(29). These studies help interpret the clinical flutamide studies by demonstrating that androgens can counteract the inhibitory effects of progesterone at the level of the central GnRH network. Further observations found that DHT implants increased voltage-gated calcium currents in GnRH neurons and, that progesterone implants eliminated this DHT-elicited increase (30). Taken together these studies suggest effects of excess androgens on GnRH neuron properties may happen both directly at the level of the GnRH neuron, likely through neuromodulatory effects, and indirectly on the central neuronal network.

In addition to endocrine disruptions, metabolic disruptions are common among PCOS patients and often include obesity, insulin resistance, and altered glucose tolerance. It is estimated that half of women with PCOS are overweight or obese (31) and that the prevalence of PCOS may be increased in overweight women (32,33). Insulin resistance coupled with hyperinsulinemia is extensive in PCOS patients. Studies in cultured theca cells, a class of ovarian cells responsible for steroid production, show that insulin stimulates increased testosterone production from cells of PCOS patients

more than in cells from control patients (34). Interestingly, not all PCOS patients that are insulin resistant are obese (35).

Potential Causes of PCOS

Both environmental and genetic factors have been postulated to underlie PCOS. PCOS has strong familial associations; women born for PCOS mothers are more likely to develop certain aspects of PCOS and 22% of sisters to women with PCOS have PCOS themselves, a percentage higher than in a randomly selected population (36). Men of the family are also affected, with sons of women with PCOS having a higher incidence of insulin resistance and an increased body weight at birth (37). Due to these strong familial associations many studies have sought to find causal genetic links behind PCOS. Recent genome-wide association studies (GWAS) have provided some insight to multiple genetic loci that may underlie PCOS(38,39). A number of these genes have been shown to be associated with reproductive perturbations found in PCOS patients, such as those for the LH and FSH receptors. Two early loci that did not have obvious reproductive links included THADA and DENND1A. Expression of a variant of the DENND1A protein was found to increase androgen biosynthesis in the ovary (40), suggesting the locus could contribute to androgen biosynthesis and thus the hyperandrogenemia found in PCOS patients. THADA has been linked to type 2 diabetes (38), which PCOS patients are at greater risk of developing. Despite initial GWAS loci being repeatedly noted in subsequent GWAS studies across different ethnic groups(41), no single gene can explain all of the anomalies present in PCOS patients. In agreement with this, studies using the classic twin model, which seek to understand if

genetic and or environmental factors may underlie the investigated trait(s), suggest that PCOS is likely polygenetic and influenced by several environmental factors (42).

Epigenetic changes brought about by genetic and/or environmental factors have also been hypothesized to play a role in PCOS. Patients with congenital adrenal hyperplasia, who have elevated androgens *in utero*, display characteristics of PCOS including cystic ovaries (43) and increases in LH-pulse amplitude (44,45). An initial study looking at global DNA methylation found no difference between PCOS and control patients (46). One downfall of this study was that this was a global analysis of peripheral blood samples that did not involve tissues specifically affected by PCOS. Epigenetic studies in specific human tissues including adipose (47) and mature oocytes (48) now support that epigenetic changes are present in women with PCOS. Additionally, one study in visceral adipose tissue of monkeys prenatally exposed to androgens noted several differences in DNA methylation between control and exposed monkeys in both infancy and adulthood (49). This study suggests that epigenetic changes that are associated with PCOS can occur in instances of *in utero* androgen exposure and that these changes can be noted in early life.

In addition to genetic and epigenetic changes, increased *in utero* androgens have been implicated as an environmental factor in driving PCOS. Studies in pregnant PCOS women have demonstrated that androgens levels are higher during mid to late gestation (weeks 22-28) (50). As previously noted, the children born to these mothers are more likely to develop PCOS and its related comorbidities within their lifetime. This suggests a potential role for altered prenatal programming in PCOS patients via exposure to androgens during certain time points *in utero*. Extensive studies in animal

models have further supported this possibility. Prenatal androgen exposure paradigms have consistently produced PCOS-like phenotypes across multiple species including monkeys(51), sheep(52), rats(53) and mice(54). Rhesus monkeys exhibit menstrual dysfunction (51), higher LH levels and lower FSH levels than in controls, and enlarged ovaries with multiple follicles present when exposed to testosterone propionate *in utero*. Sheep prenatally exposed to testosterone possess similar phenotypes and also display differences in adipocytes (55) and disrupted insulin signaling (56). Similar changes to those found in sheep could contribute to metabolic disruptions found in PCOS patients. In rats, prenatal exposure to testosterone or its non-amortizable form, 5-alpha-dihydrotestosterone (DHT), suppressed estradiol-induced LH surges, increased LH pulse frequency, and suppressed progesterone receptor gene expression in the preoptic area (53). As previously discussed, LH pulses in women with PCOS are less sensitive to progesterone feedback and androgens likely play a role in this impairment. Progesterone negative feedback inhibits GnRH neuron firing while androgens increase it (57). Suppression of progesterone receptor gene expression in the POA, where GnRH neurons reside, could thus account for the reduced neuronal sensitivity to progesterone feedback in PCOS women.

Prenatally androgenized (PNA) mouse models: a window into GnRH neuron physiology

The PNA mouse model recapitulates many aspects of PCOS. This mouse model is produced by subcutaneous injection of DHT during gestational days 16-18. Female mice born from DHT-injected mothers display several reproductive deficits similar to PCOS patients including increased LH, LH pulse frequency, testosterone levels, and disrupted reproductive cyclicity (58,59). Also, much like in PCOS women, studies in

PNA mice demonstrate changes in ovarian morphology including a decrease in corpora lutea (60). The presence of these PCOS-like phenotypes in PNA mice makes this mouse model ideal for investigating underlying neuroendocrine mechanisms driven by excess androgens *in utero* which are present in PCOS. A study in ovariectomized PNA mice demonstrated that while PNA mice are capable of having a preovulatory LH surge (estradiol positive feedback), they do possess impaired negative feedback to estradiol (60). Also progesterone replacement in ovariectomized (OVX) PNA mice did not decrease LH levels as in control mice, and expression of progesterone receptor was decreased in PNA mice in brain areas implicated in steroid feedback such as the anteroventral periventricular (AVPV) and hypothalamic arcuate nuclei, which house kisspeptin neurons that strongly excite GnRH cells and are involved in mediating steroid feedback (59). These studies provide potential mechanistic explanations for clinical findings discussed above that indicate that feedback mechanisms are altered via androgens in PCOS. Further, they point to a role of early excess androgen exposure in driving these changes. Interestingly, in PNA mice, the androgen receptor antagonist flutamide improved reproductive cyclicity (58) and increased recruitment of preovulatory follicles (61). Thus blocking androgen action via flutamide can improve PNA phenotypes similar to improving aspects of PCOS in clinical studies.

Due to the strong prevalence of metabolic complications in women with PCOS, studies in PNA mice have also examined if prenatal androgen exposure can contribute to metabolic abnormalities observed in PCOS patients. Metabolic studies in PNA mice, however, demonstrated no difference in body mass or insulin resistance, but impaired glucose tolerance was observed (54). This impaired glucose tolerance was attributed to

impaired function of pancreatic islet cells. Follow up *in vitro* studies demonstrated that the presence of androgens impaired the calcium increase in islets cells to glucose. These observations suggest that some metabolic complications in PCOS may be driven by androgens independently of other metabolic anomalies such as obesity. Supporting this idea, one study in women with PCOS observed that beta cell dysfunction is independent of both glucose tolerance and obesity (62). Also, much like some PCOS patients, reproductive cyclicity in PNA mice is improved via metformin (63) and wheel running (increased voluntary exercise) (64). Interestingly, cyclicity was improved via wheel running even without any weight loss, suggesting the effects of exercise on cyclicity may be independent of the metabolic axis.

Because studying neuronal aspects of PCOS are not possible in patients, PNA mice have also given investigators a window by which to examine possible neuronal underpinnings of PCOS. Electrophysiologic recordings in acutely-prepared brain slices from PNA mice have given insight to neuronal changes underlying reproductive and neuroendocrine abnormalities. Firing rate of GnRH neurons is increased in adult female PNA mice (63). This firing likely drives hormone release as in other neuroendocrine cells (65). Increased GnRH neuron firing in PNA mice, as well as increased LH, was reversed by the AMP-activated protein kinase (AMPK) activator metformin, despite little to no effect of PNA on metabolic parameters(63). These results again suggest a direct effect on the reproductive axis is possible, rather than an effect through altered metabolism. This finding coincides well with the findings discussed above that demonstrate few metabolic perturbations are present in PNA mice.

One likely driver of increased GnRH activity in PNA mice is the upregulation of GABAergic inputs to GnRH neurons (58). GABAergic input to GnRH neurons is depolarizing and can be excitatory due to an increased intracellular chloride concentration in these cells (66). Thus, increased GABAergic input in PNA mice leads to cell membrane depolarization and more action potential firing. This increased firing could contribute to increased GnRH pulse generator function in PNA mice. Studies also demonstrated that increases in GABAergic input were action potential independent, suggesting the increased GABA input to GnRH neurons is likely due to an increase in number of release sites (58) in PNA mice. This finding was further supported by morphological studies demonstrating that GnRH neurons in PNA mice have a greater spine density that correlates with GABA synapses, and more contact with GABA neurons from the arcuate nucleus (59). Interestingly, androgens increase spine density in other brain areas (67). This suggests that androgens may alter the structure and function of GnRH neurons and that the afferent neuronal network to these cells are altered by PNA. As mentioned above, blocking androgen action via flutamide improved aspects of PCOS in clinical studies and gross reproductive parameters in PNA mice. Flutamide also improved neuronal deficits found in PNA mice. Subcutaneous flutamide injections decreased functional GABAergic input to GnRH neurons to control levels, reverse increased GABAergic postsynaptic current amplitude and frequency (58), and reverse morphological increases in GABAergic spines on GnRH neurons in PNA mice (61). These studies support both central and downstream reproductive changes in PNA mice are driven via action of androgens and, further, that they are likely attributable at

least in part to increases in GABA inputs to GnRH neurons, perhaps arising in the arcuate region.

Reproductive activity prior to puberty

The timing of PCOS onset has been difficult to ascertain due to a lack of observable reproductive indicators prior to puberty, as well as ethical issues concerning the measurement of certain PCOS parameters in adolescent females (for example transvaginal ultrasound to observe ovaries). As a substitute, obese girls, who often exhibit hyperandrogenemia, have been studied (68,69). This work suggests that neuroendocrine and metabolic changes can arise prior to and/or across pubertal development and also indicate a role for androgens in driving development of adult PCOS. One study suggested premenarchal girls with hyperandrogenemia have a tendency for increased LH pulse frequency, however small numbers in this clinical study did not allow for significance to be observed between groups (70). Daytime LH pulse frequency is increased in obese young women with PCOS, however patients in this study had no evidence of hyperandrogenemia (71). This suggests that early LH pulse frequency may be programmed, rather than being an effect of the presence of increased androgens. Hyperandrogenemia in adolescent girls was improved by metformin but metformin did not change hypothalamic response to progesterone feedback (72). This is similar to results in adults in which metformin did decrease testosterone levels but did not reduce LH pulse frequency in the presence of progesterone and estradiol (73). This suggests that effects of androgens on hypothalamic regulation may be independent of effects of progesterone in girls, many of whom have very low progesterone because they have not commenced cycles.

Studies in monkeys also support that adult altered reproductive neuroendocrine function can be programmed via prepubertal exposure to excess androgens. Monkeys that were treated with a 3.7 fold increase in testosterone, similar to the elevation in women with PCOS, via implants beginning at 1 year of age had increased LH pulse frequency in the early follicular phase and an increased response to GnRH in early (at 4yrs) but not later (5yrs) time points (74). These studies suggest prepubertal presence of testosterone can program an upregulation of the reproductive axis that appears later in life. Thus neuroendocrine aspects of reproduction can be altered via effects of androgen exposure before puberty. This suggests that reproductive function at the level of the brain occurs and can be altered before outward signs of puberty are present.

During the postnatal time period, LH and FSH levels are relatively low, with the exception of the minipuberty of infancy that is primarily observed in primates (75). In mice, testosterone rises in males only at 4 hours but not at 20 hours after birth, and this increase is independent of both kisspeptin and GnRH (76,77). This however does not rule out that GnRH release and activity could be occurring at a central level. An innovative set of studies in which a method to measure GnRH release in brain slices was developed, suggest that GnRH release is indeed occurring during the postnatal time period in male mice and that the level of this release was higher than in adulthood (78). Increased GnRH release at younger ages was not coupled with a pituitary LH release in response to a GnRH challenge, despite adequate mRNA expression for gonadotropin subunits and GnRH receptor. This suggests that while downstream reproductive function is not yet active upstream reproductive function is occurring.

Neuroendocrine release is thought to be coupled to activity. Thus if early GnRH release is occurring, GnRH neurons and their inputs are likely active. Early neuronal activity in other physiological systems allows for successful neuronal wiring to later drive physiological function. For example, changes in activity of the visual system have been shown to drive synapse formation and can alter visual outcomes when disrupted (79). Prior to this dissertation, however, few functional studies had been performed to ascertain directly if the GnRH neuronal network is active prior to the time of pubertal maturation and what may be the role of such prepubertal activity.

Dissertation preview

The studies that follow are the first direct examination of activity of GnRH neurons before puberty. The following chapters focus on postnatal GnRH neuron activity in control and PNA mice. Chapter 2 characterizes prepubertal GnRH neuron activity in both control and PNA mice in both sexes. Chapter 3 examines the role of ovarian factors in GnRH firing in both adult and prepubertal PNA mice. Chapter 4 focuses on the role of postnatal GnRH neuron activity in adult reproductive function. To conclude, chapter 5 summarizes these findings, discusses their possible implications in the larger scope of this field of research, and identifies possible directions for future studies. Together, this work gives insight to early reproductive activity in both normal and reproductively altered conditions and how such activity may impact later reproductive function.

Chapter 2 Prepubertal development of gonadotropin-releasing hormone (GnRH) neuron activity is altered by sex, age and prenatal androgen exposure

Co-authored with Suzanne M. Moenter

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Abstract

Gonadotropin-releasing hormone (GnRH) neurons regulate reproduction though pulsatile hormone release. Disruption of GnRH release as measured via LH pulses occurs in polycystic ovary syndrome (PCOS), and in young hyperandrogenemic girls. In adult prenatally androgenized (PNA) mice, which exhibit many aspects of PCOS, increased LH is associated with increased GnRH neuron action potential firing. How GnRH neuron activity develops over the prepubertal period and whether or not this is altered by sex or prenatal androgen treatment are unknown. We hypothesized GnRH neurons are active before puberty, that this activity is sexually differentiated and altered by PNA. Dams were injected with DHT on d16-18 post copulation to generate PNA mice. Action potential firing of GFP-identified GnRH neurons in brain slices from 1, 2, 3, 4 week-old and adult mice was monitored. GnRH neurons were active at all ages tested. In control females, activity increased with age through 3wks, then decreased to adult levels. In contrast, activity did not change in PNA females, and was reduced at 3wks. Activity was higher in control females than males from 2-3wks. PNA did not affect GnRH neuron firing rate in males at any age. Short-term action potential patterns were

also affected by age and PNA treatment. GnRH neurons are thus typically more active during the prepubertal period than adulthood, and PNA reduces prepubertal activity in females. Prepubertal activity may play a role in establishing sexually-differentiated neuronal networks upstream of GnRH neurons; androgen-induced changes during this time may contribute to the adult PNA, and possibly PCOS, phenotype.

Précis

GnRH neurons, which ultimately control fertility, are surprisingly most active before puberty. This activity is reduced in females exposed in utero to androgens, potentially altering the GnRH neuronal network and adult function.

Introduction

The ability to reproduce is typically associated with maturation into adulthood. The mechanisms underlying the establishment of the reproductive axis prior to and during puberty—and how disruptions of such mechanisms may be linked to various types of infertility—are not fully understood, particularly at the neural level. GnRH neurons link the central nervous system to the peripheral reproductive system through the release of GnRH. GnRH stimulates anterior pituitary secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which activate gonadal functions; gonadal hormones exert feedback to control GnRH release and pituitary response. GnRH is released in pulses (8,80) with higher frequency favoring LH synthesis and release and lower frequency favoring FSH (10,81). Disruption of pulsatile GnRH release or interfering with the typical modulation of pulse frequency during the reproductive cycle can lead to infertility (82). An example of the latter is polycystic ovary syndrome (PCOS), the leading cause of infertility in women (82). Many women with PCOS exhibit persistently

high GnRH-pulse frequency as reflected by LH pulses in the peripheral circulation (83-85). This exacerbates hyperandrogenemia and produces a gonadotropin milieu that does not promote effective follicle maturation and ovulation.

To study neurobiological mechanisms potentially contributing to PCOS, prenatally androgenized (PNA) animal models are commonly used; women with PCOS who do achieve pregnancy exhibit elevated androgen levels in late gestation, potentially exposing their offspring to androgen excess during development (50,86). In several species, PNA females exhibit many neuroendocrine aspects of PCOS, including disrupted cycles and elevated LH pulse frequency, as well as mildly increased testosterone levels (51-54,58,87). In adult female PNA mice, GnRH neuron action potential firing activity is increased (63), as is GABAergic synaptic transmission to these cells; GABA can excite GnRH neurons to fire action potentials (58,66). These studies in PNA mice have revealed possible mechanisms contributing to the neuroendocrine phenotype in adults, but have not addressed when programming-induced changes become evident during development. These early changes are potentially relevant to the human condition because clinical studies suggest altered LH release manifests before the pubertal transition is complete in hyperandrogenemic girls (70) Additionally, late pubertal (Tanner stage 4-5) girls with hyperandrogenemia exhibit increased LH pulse frequency (71,88) and girls diagnosed with PCOS have increased LH pulse frequency as young as 12 years of age (71). A role for androgens in this increased pulse frequency is suggested by studies in rhesus macaques, in which mild elevation of testosterone, characteristic of PCOS, during pubertal development increases LH pulse frequency during the early follicular phase (74). In prepubertal control mice,

gonadotropin levels are lower than adults (78,89,90). In contrast, studies measuring GnRH release in brain slices from male mice indicate GnRH release frequency is highest during the first week of postnatal life (78). This discrepancy is potentially explained by the well-established down regulation of pituitary response to GnRH when exposed to high frequency pulses or continuously high levels of this hormone (9,10). These observations suggest GnRH neurons are functional before puberty; that is before downstream activation of the reproductive system is evident.

In these studies, we characterized GnRH neuron activity during prepubertal development in control and PNA mice. We hypothesized that GnRH neurons are active before puberty and that PNA increases this activity. To examine sex differences in typical development as well as to determine if effects of PNA are similar to those of typical masculinization, both females and males were studied.

Methods

Chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless noted.

Animals GnRH-GFP mice on a C57Bl6/J background were held on a 14L:10D light cycle with lights on at 0300 Eastern Standard Time (EST). Mice had *ad libitum* access to water and chow (Teklad 2916) except breeders, which received higher protein 2919 chow (both from Envigo, Madison, Wisconsin, USA). To generate PNA mice, a GnRH-GFP and a CD1 female were paired for 1-3 wks and then a stud male introduced. Males were removed after pregnancy was established. PNA mice were generated by injecting pregnant GnRH-GFP dams with DHT (225 µg sc in sesame oil vehicle) on d16–18 of

gestation (d1, copulatory plug observed). Controls included mice from vehicle (VEH)-treated or uninjected GnRH-GFP dams; no differences were observed between control types and they were combined. The CD1 mouse was included for maternal and nutritional support to increase survival of PNA pups. Litter sizes were adjusted to <15 pups per cage (two lactating females; CD1 and GnRH-GFP dam in each cage) by culling CD1 pups to normalize nutrition. Pups not used for postnatal recordings were weaned at 3 wks of age. Androgenization of PNA females was examined by measuring time of vaginal opening, anal-genital distance, and estrous cycles by vaginal lavage for 15 consecutive days in remaining female littermates in adulthood; preputial separation was monitored in males. Because of the strict age requirements for recording pups, pups from 4-9 litters were used. For adult recordings, 2-5 litters were used as these were recapitulating previous observations (29,57,58). The Institutional Animal Care and Use Committee of the University of Michigan (PRO00006816) approved all animal procedures preformed in this study.

Slice preparation. All solutions were bubbled with 95% O_2/5% CO_2 throughout the experiments and for at least 30 min before exposure to tissue. Between 0550-1530 EST, the brain was rapidly removed and placed in ice-cold sucrose saline solution containing (in mM): 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 NaH_2PO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal (300 μm) slices were cut with a Leica VT1200S (Leica Biosystems Buffalo Grove, IL, USA). Slices were incubated in a 1:1 mixture of sucrose saline and artificial cerebrospinal fluid (ACSF) containing (in mM): 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO4, 1.2 $MgSO_4$, 2.5 $CaCl_2$ (pH 7.4) for 30 min at room temperature (21- 23 ºC) and then transferred to 100% ACSF for

additional 30 min at room temperature before recording. Recordings were performed 1-5 h after brain slice preparation; no difference in firing patterns were evident based on time after brain slice preparation or time of day.

Extracellular recording. For recording, slices were placed into a chamber continuously perfused with ACSF at a rate of 2-3ml/min with oxygenated ACSF heated to 30-32 ºC with an inline-heating unit (Warner Instruments, Hamden, CT, USA). GFP-positive GnRH neurons were identified by brief illumination at 488 nm on an Olympus (Center Valley, PA, USA) BX51WI microscope. Recording micropipettes were pulled from borosilicate capillary glass (type 7052, 1.65 mm outer diameter; 1.12 mm inner diameter; World Precision Instruments, Inc. Sarasota, FL, USA) using a Flaming/Brown P-97 puller (Sutter Instruments, Novato, CA USA) to obtain pipettes with a resistance of 2-3 MΩ when filled with HEPES-buffered solution containing (in mM): 150 NaCl, 10 HEPES, 10 glucose, 2.5 CaCl2, 1.3 MgCl2, and 3.5 KCl. Recordings were made with an EPC-8 with ITC-18 interface or one channel of an EPC-10 dual patch clamp amplifier using Patchmaster software (HEKA Elektronik, Pfalz, Germany) running on a Macintosh computer. Low-resistance (<50 MΩ) seals were formed between the pipette and neuron after first exposing the pipette to the slice tissue in the absence of positive pressure. Recordings were made in voltage-clamp mode with a 0 mV pipette holding potential. Signals were acquired at 10 kHz and filtered at 5 kHz. Resistance of the loose seal and baseline was checked during an initial 5-15 min stability period, and also at 10-min intervals during recording; data were not used if seal resistance increased above 50 MΩ.

Experimental design Brain slices were prepared from control and PNA female and male mice at 1, 2, 3, 4 weeks of age and adults (17-38 weeks of age). Three-week old mice were utilized before weaning to avoid changes subsequent to abrupt social and nutritional changes. Targeted extracellular recordings were used to record long-term (20-60min) patterns of firing activity. This method maintains internal milieu and has minimal impact on the firing rate of neurons (91,92). Recorded cells were mapped to an atlas (93) to determine if any trends based on anatomical location emerged; no such trends were apparent in these data sets. No more than three cells per animal and two cells per slice were included for analysis, and at least four mice were tested per group. Variation within an animal was not less than among animals.

Analysis Action currents (events) were detected off-line using custom programs in Igor Pro 6.31 (Wavemetrics, Lake Oswego, OR USA). Data were binned at 60-sec intervals and were transferred to Excel. Mean firing rate (Hz) was calculated by dividing the total number of events by the duration of recording. Further pattern analyses were done for the 3wk-old and adult groups, in which the most striking differences in mean firing rate were observed. The distribution of interspike intervals (ISI) for each group was examined by comparing the probability of occurrence of ISIs between 0.01 and 100s in increments of 0.5s. A histogram of ISIs for all cells within a group was constructed and these distributions normalizing by dividing each interval by the total number of ISIs for the group. Normalized ISI distributions were compared using a Kolmogorov- Smirnov test.

Short-term burst-firing patterns are typically associated with neurosecretion (65). Burst parameters (duration, spikes/burst, frequency, intraburst and interevent intervals), were

compared among groups. Bursts have been characterized in adult GnRH neurons (94-96), but given the difference in firing rate and patterns observed in the present study between prepubertal and adult mice, previously described burst parameters may not be optimal to analyze at bursting activity at all ages. Bursts were thus first detected using software that systematically adjusted the maximum time between events (burst window) for inclusion in a burst (10 ms intervals up to 2 s total duration). The number of bursts was graphed as function of burst window. In most groups, a distinct peak was observed revealing a burst window that yielded the maximum number of bursts. This burst window was used for further analysis. Two groups (adult PNA females and males) did not exhibit a distinct peak, rather the number of bursts detected increased gradually as the burst window was lengthened; these groups were included in the analysis of burst parameters but since they lacked a clear peak, were not used to select the burst window for further analysis. The peak of the burst window (range 0.36 to 0.78s) for each group exhibiting a peak was used to calculate burst parameters for all groups. One-way ANOVA revealed that the same statistical differences were found for burst frequency among groups regardless of the burst window chosen within this range ($p>0.99$). We therefore chose a burst window of 0.36 (max burst number for 3-wk old control females) to compare burst parameters among age, and treatment groups by sex.

Statistics. Statistical analyses were performed using Prism 7 (GraphPad Software, La Jolla, CA, USA) and IBM SPSS version 22 (IBM Corp., Armonk, NY, USA). Data are reported as individual values with mean±SEM. Data distributions were tested using Shapiro-Wilk normality test and used along with experimental design to select appropriate statistical comparisons. For within-sex comparisons, two-way ANOVA was

used to compare all groups with Fisher's least significant difference *post hoc* test; this choice was justified by the large number of comparisons (i.e., 45 unique comparisons within each sex between both treatments at five different ages). When comparisons of were restricted to between groups at the same age, Sidak was used a post hoc. Burst parameters for each sex were compared for effects of age and treatment with two-way ANOVAs with Tukey's HSD test, Tukey's was chosen for this analysis to permit comparisons of the mean of each group to all others. Further tests are specified in the figure legends and results. The null hypothesis was rejected if $p<0.05$, but all p values <0.1 are reported.

Results

Prenatal androgenization alters timing of pubertal indicators in both sexes and reproductive parameters in adult female mice. PNA females exhibit early vaginal opening, and altered hormone levels and disrupted cycles as adults (59,63,97). Here, we studied when PNA-induced differences emerge with postnatal development, often precluding evaluation of these parameters in the study subjects. These aspects were thus verified in littermates that were allowed to survive to adulthood; in our experience, the PNA phenotype is consistent among littermates. To verify the prenatal androgenization phenotype, vaginal opening in females and preputial separation in males were monitored from weaning. As reported (54), vaginal opening occurred at a younger age in PNA females despite a lower body mass, which was attributable to younger age (Figure 2-1A, B, n= 26 control and 30 PNA, age at VO $p < 0.0001$ two-tailed Mann Whitney, body mass at VO $p<0.0001$ two-tailed unpaired Student's t test for samples with similar standard deviations). In males, preputial separation was advanced

in PNA mice but no difference in body mass was observed, likely because the absolute shift in age of preputial separation for males was only ~2 days (Figure 2-1C,D, n= 11 control and 6 PNA, both $p < 0.05$ two-tailed unpaired Student's t-test for samples with similar standard deviations). PNA treatment increased anogenital distance in females (Figure 2-1E stats), and also disrupted cyclicity in adult littermates of PNA mice used for recordings, (Figure 2-1F). Specifically, PNA decreased the percent of days in estrus and proestrus (n=15 both control and PNA; estrus control 29±1.6%, PNA7±1.8%, proestrus control 15±1.4%, PNA 1±0.75%, both $p<0.0001$, Chi-squared test). The outward effects of PNA are thus present by puberty in both sexes and persist in females into adulthood.

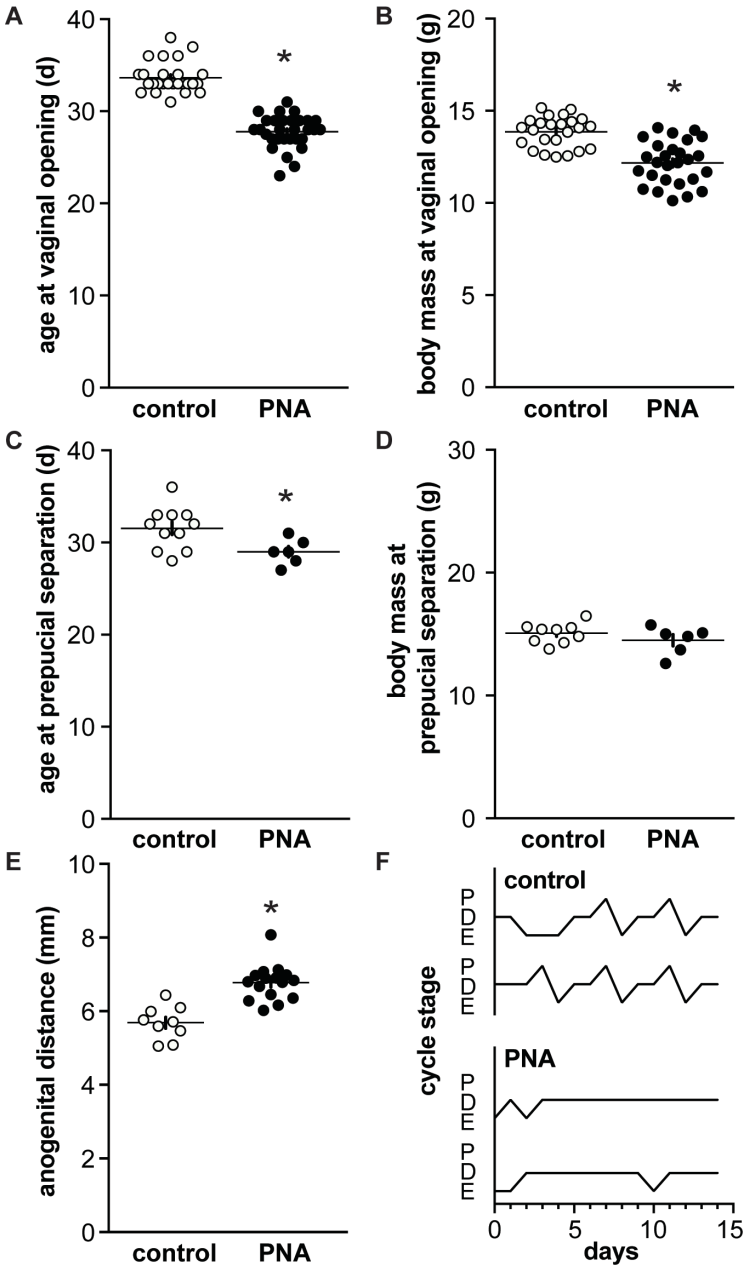

Figure 2-1. Characterization of PNA animals. A, B) Age (A) and body mass (B) at vaginal opening (VO) in control and PNA females. C, D) Age (C) and body mass (D) at preputial separation (PS) in control and PNA males. E) Anogenital distance in adult female littermates of mice that were used for recording before puberty. F) Representative estrous cycles in adult female littermates: P, proestrus; D, diestrus; E, estrus. * $p<0.05$ control vs PNA, unpaired Student's t-test in all but (A) in which a Mann Whitney was used.

Prepubertal development of GnRH neuron activity is sexually differentiated. To test the hypotheses that GnRH neurons are dynamically active through prepubertal development, extracellular recordings were made of GFP-identified GnRH neurons. Representative traces from female mice are shown in Figure 2-2, summary data in Figure 2-3, and statistical parameters in Tables 2-1 and 2-2. In cells from control females, firing activity was low during at one week of age, and increased each week to a peak at three weeks of ages, before declining to adult levels. In contrast to females, GnRH neuron activity in control males decreased from the first to second postnatal week of age, then rose again at week three before returning to lower levels during adulthood (Figure 2-3A). As a result of these different developmental patterns, activity was greater in control females than control males at two and three weeks of age. GnRH neuron firing activity during prepubertal development is thus both notably higher than adults and is sexually differentiated in control mice.

PNA alters the development of GnRH neuron activity in both sexes. Based on observations of increased reproductive neuroendocrine activity in both women with PCOS and PNA animal models, we hypothesized that PNA increases GnRH neuron firing in female mice during the prepubertal period. Contrary to our hypothesis, firing rate was lower in PNA mice at three weeks of age and the p value approached the level set for significance at two weeks of age. Activity did not change with age in cells from PNA females over the period examined (Figure 2-3B, $p \geq 0.097$). In utero exposure to androgens thus alters the normal development of GnRH neurons in females, reducing firing activity during the prepubertal period rather than increasing firing as postulated.

In contrast to cells from PNA females, activity of cells from PNA males varied with age. Specifically, activity of GnRH neurons from male PNA mice was decreased at three weeks of age and in adults compared to one-week-old mice (Figure 2-3C). Of note, PNA treatment partially ameliorated the decline of activity from one to two weeks of age that was observed in control males. Also in contrast to females, there was no difference in GnRH neuron activity between control and PNA males of the same age, suggesting that effects of PNA treatment in male offspring may be milder than in female offspring.

Prepubertal development of GnRH neuron activity in PNA females is similar to that of control males. To examine if PNA treatment in females results in a GnRH neuron activity development pattern that is similar to males, we compared recordings between PNA female and control male mice at all ages tested. There was no difference in GnRH neuron activity between PNA female and control males at any age ($p>0.3$, two-way ANOVA/Sidak, Figure 2-3D; $p>0.08$ two-way ANOVA/Fisher's LSD, not shown). These data suggest PNA treatment in females at least in part masculinizes development of GnRH neuron activity.

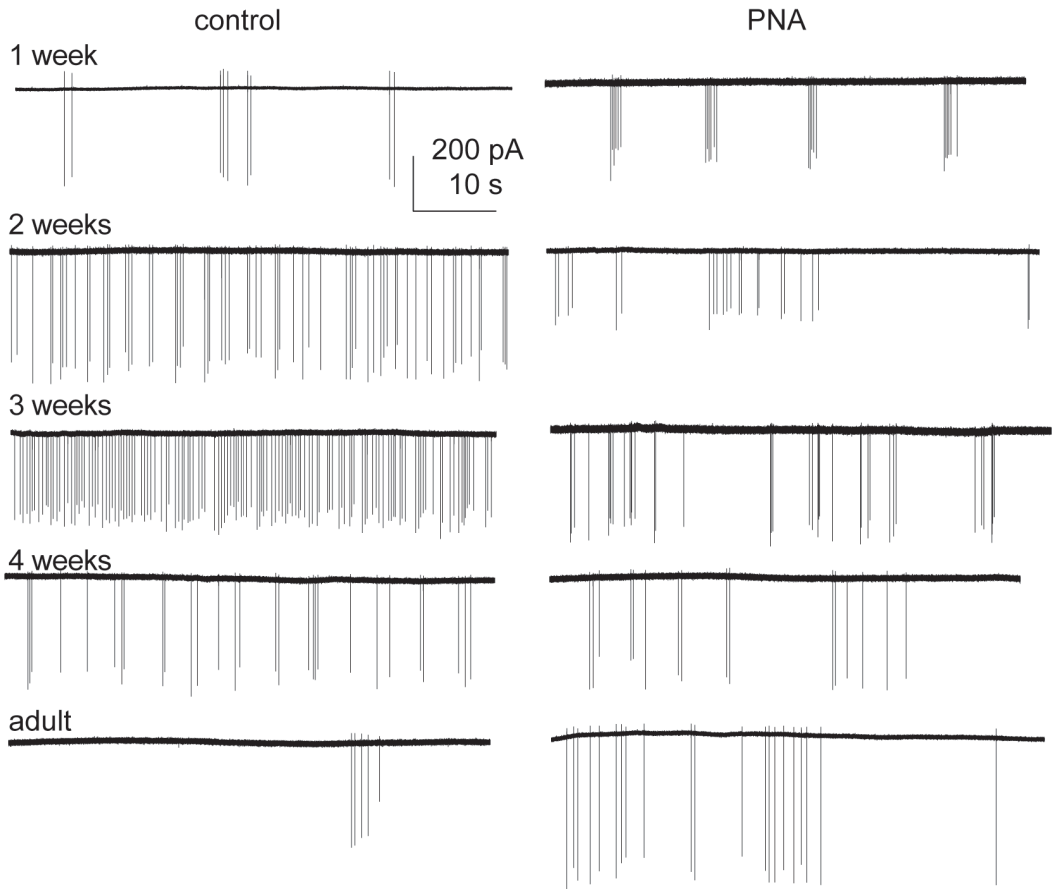

Figure 2-2. Activity of GnRH neurons from female mice changes with age and prenatal androgenization. One-minute representative raw recordings of GnRH neurons from control (left) and PNA (right) females.

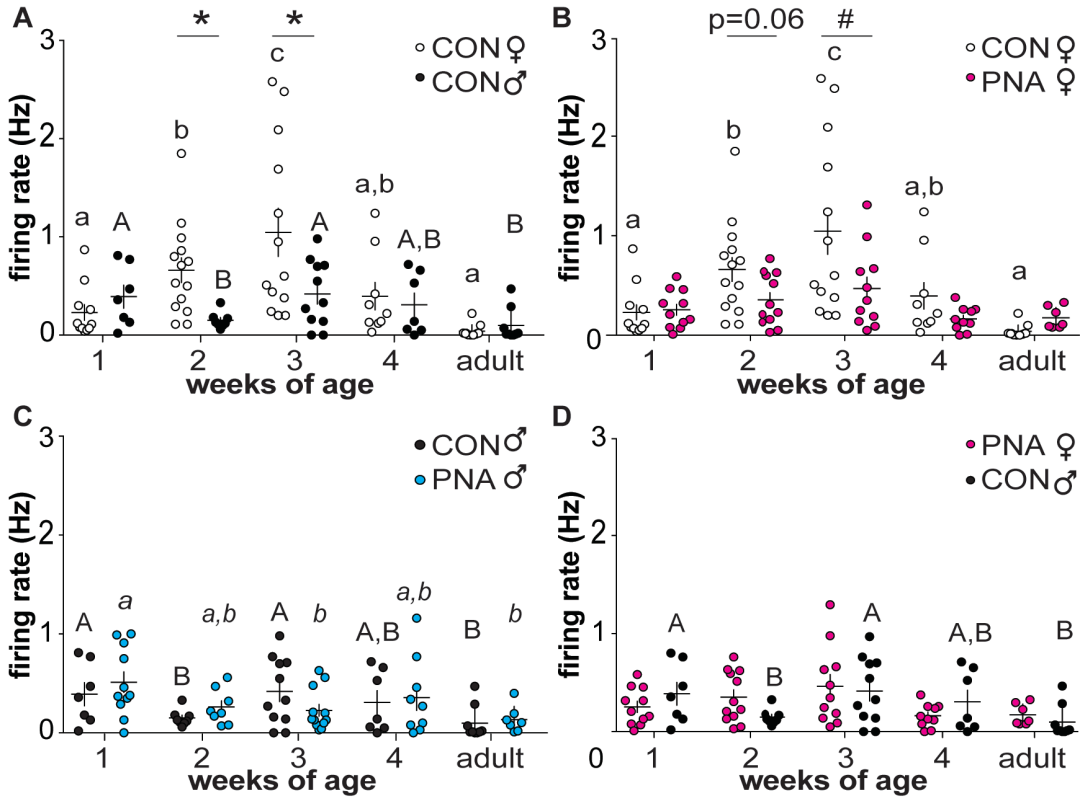

Figure 2-3. GnRH neuron activity changes throughout the prepubertal period in both sexes and is altered by PNA. A-D, Individual values and mean ± SEM of firing rate at 1, 2, 3, 4 weeks of age and adults. A, control females (open circles) and males (black circles); B, control and PNA (magenta circles) females; C, control and PNA (blue circles) males; D, PNA females and control males. Different letters of the same case indicate differences with age within a group; there were no changes with age in female PNA mice ($p \geq 0.0978$). * $p < 0.05$, # $p < 0.01$ between treatment groups at each age. Two-way ANOVA/Fisher's LSD for comparisons among all groups within sex; two-way ANOVA/Sidak for comparisons at the same age between sexes (control female vs control male; PNA female vs control male). CON, control.

Table 2-1. Cells/group for activity recordings.

age (wks)	CON ♀	PNA ♀	CON ♂	PNA ♂
1	11	11	7	11
2	14	12	8	8
3	13	11	11	12
4	9	10	7	9
adult	11	7	9	7

Table 2-2. Two-way ANOVA parameters for comparison among firing rates within groups

Comparison (Figure)	Age	Sex	Interaction
♀ con vs ♂con (3A)	F(4,90)=6.613***	F(1,90)=5.091*	F(4,90)=3.304*
	Age	**Treatment**	**Interaction**
♀ con vs ♀PNA (3B)	F(4,99)=8.074***	F(1,99)=5.464*	F(4,99)=2.405
♂ con vs ♂ PNA (3C)	F(4,79)=3.712**	F(1,79)=0.1815	F(4,79)=1.139
	Age	**Sex & Treatment**	**Interaction**
♀ PNA vs ♂ con (3D)	(F4,83)=3.724**	F(1,83)=0.0388	F(4,83)=1.504

*p < 0.05; **p < 0.01; ***p < 0.001

Action potential timing in GnRH neurons changes with age, sex and PNA treatment.

The above firing rate data are mean values over recordings lasting up to one hour. This provides a good overview of activity levels, but differences in spike timing are also important to neuronal function, in particular for the release of neuropeptides in adults (65,98) and synapse formation during development (99). We investigated action potential patterning of GnRH neurons from mice of each sex and treatment at three weeks of age and in adults as these ages had the largest PNA-induced difference in overall activity in females, which are of primary interest with regards to PCOS. First, we

compared the probability distribution of interspike intervals (ISI) among groups. In histograms of ISI probability, a sharp peak indicates a fairly regular firing pattern, multiple peaks indicate strict burst firing (long interval between bursts and shorter intervals within bursts) and flatter distributions indicate irregularly firing neurons that may exhibit a combination of bursts and individual spikes (100); ISI distributions for most groups of GnRH neurons studied fell into this latter category. In control females, ISI distributions shifted towards longer intervals in adults (Figure 2-4A, Kolmogorov-Smirnov (KS), $p<0.0004$, the weeks vs adult; note KS tests only permit two distributions to be compared). There was no difference between PNA and control females at three weeks of age (KS, $p=0.3927$). In adulthood, however, PNA mice exhibited a longer interval shoulder that resulted in a shift in ISI distributions compared to controls (KS, $p<0.05$). These data suggest that spike intervals shift with age in female mice and that PNA treatment leads to altered ISI distribution in adults.

In control males, ISI distributions did not quite differ with age (Figure 2-4B, KS, $p=0.0522$, three week vs adult), trending towards longer intervals in adults, but did differ with PNA treatment in both three-week old (KS, $p= 0.0004$) and adult mice (KS, $p=0.0062$). Further, a distinct shoulder appeared at longer intervals in both control and PNA adult males (arrow in Figure 2-4B), suggesting emergence of a greater percentage of burst firing with age. These results indicate that despite a lack of effect of PNA on mean firing rate in males, this treatment does affect short-term organization of spikes. To further address the question of whether or not PNA induces masculinization of GnRH neuron firing in females, we compared ISI distributions between cells from PNA female and control male mice. These distributions did not differ at three weeks of age

(KS, p=0.27) but a difference emerged in adulthood (KS, p=0.03, Figure 2-4C). PNA treatment thus induces changes in spike organization in females that are distinct from masculinization

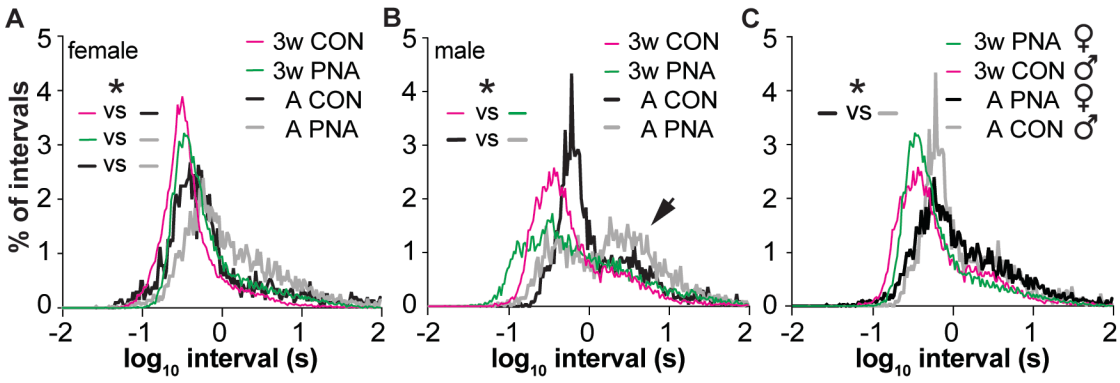

Figure 2-4. Spike timing in GnRH neurons changes with age in females and differs with PNA treatment in adults. A) Log_{10} ISI distributions of GnRH recordings for each group of female mice. B) Log_{10} ISI distribution for each group of male mice. Arrow indicates longer interval shoulder (see text). C) Log_{10} ISI distributions comparing male control and female PNA mice, * $p<0.05$, Kolmogorov-Smirnov. Note change in color panel (C) vs (A) and (B) for adult animals.

Burst patterning in GnRH neurons is affected by age but not PNA treatment. We also examined how sex age and PNA treatment affect organization of action potentials into bursts (Figure 2-5A, Tables 2-3, 2-4). In control female mice, burst frequency was higher at three weeks of age than in adults (Figure 2-5B, two-way-ANOVA/Tukey, $p<0.05$). Burst duration and spikes/burst approached significance with age in control mice, with younger animals having a longer bursts with more spikes (Figure 2-5C, D: two-way ANOVA/Tukey, p=0.0577 burst duration, p=0.0507 spikes/burst). In females, intraburst interval did not differ among groups, but interevent interval differed with age in controls, and between adult control and PNA groups (Table 2-3). In males, burst

frequency was increased in three-week-old control animals compared to adults (Figure 5E, two-way ANOVA/Tukey, $p<0.05$), however, while there was an effect with age on burst duration and spikes/burst, post hoc analysis revealed no differences among groups (Figure 2-5F, G, $p>0.16$ for all comparisons). Intraburst interval and the interevent interval were not different among male groups, and no effect of PNA was detected. Burst frequency is thus higher at younger ages in both sexes; in females the properties of these bursts are also shifted with age.

Table 2-3. Mean±SEM intraburst and interevent intervals.

Parameter	3 wk ♀ CON	3 wk ♀ PNA	3 wk ♂ CON	3 wk ♂ PNA	Adult ♀ CON	Adult ♀ PNA	Adult ♂ CON	Adult ♂ PNA
Intraburst interval (s)	0.26 ±0.01	0.27 ±0.01	0.26 ±0.01	0.24 ±0.01	0.27 ±0.02	0.27 ±0.01	0.27 ±0.02	0.26 ±0.02
Interevent interval (s)	2.86** ±0.58	8.43 ±3.35	71.2 ±67.6	16.4 ±5.11	62.1 ±19.9	14.3* ±3.90	163.4 ±117.6	46.9 ±29.0

*p<0.05; **p<0.001 vs adult control females, two-way ANOVA/Tukey

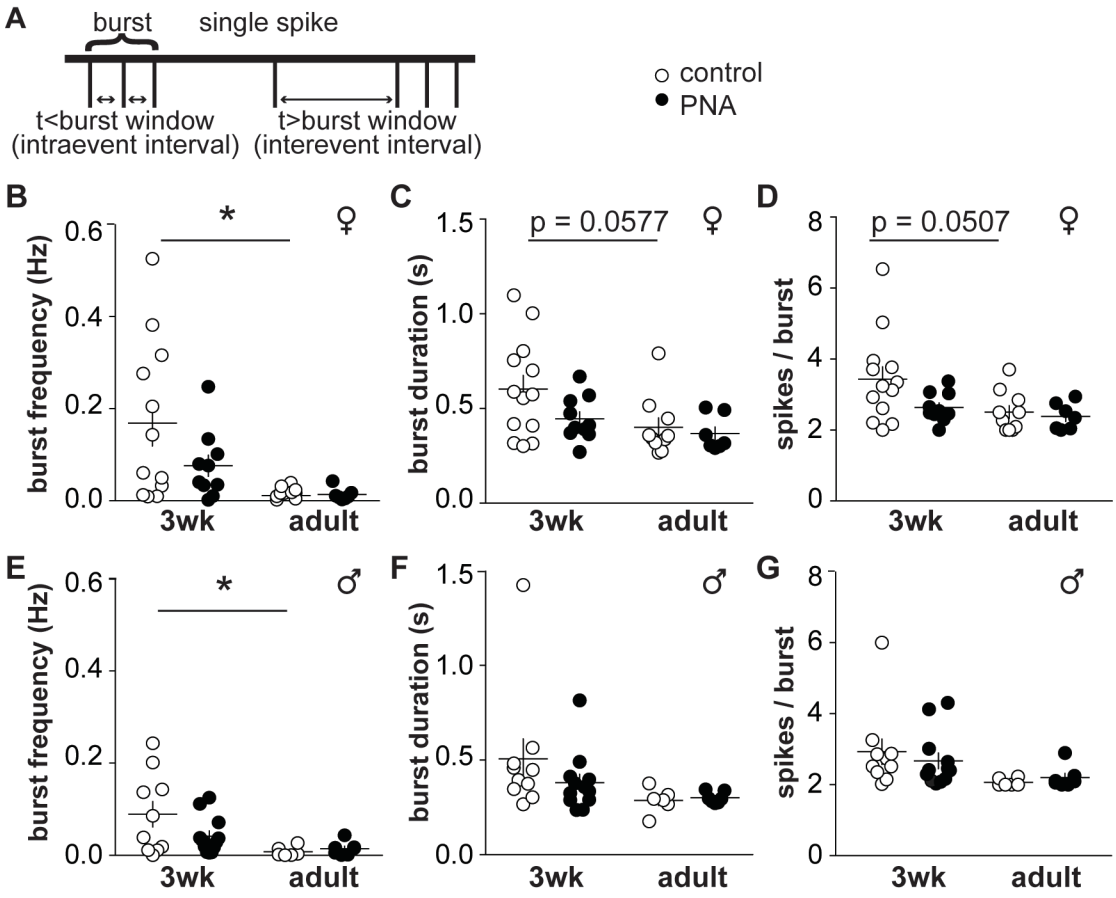

Figure 2-5. Burst patterns of GnRH neurons differ with age but are not affected by PNA. A) Schematic showing burst parameter determination. B-D Burst frequency (B), duration (C), spikes/burst (D) in cells from female mice. E-G Burst frequency (E), duration (f), spikes/burst (G) in cells from male mice. *p<0.05, two-way-ANOVA/Tukey.

Table 2-4: Statistical parameters for burst analysis

Comparison	Parameter	Age	Treatment	Interaction
♀ CON vs ♀PNA	frequency	F(1,38) =11.72**	F(1,38)= 2.522	F(1,38)= 2.867 p=0.09
	duration	F(1,36) =5.612*	F(1,36)= 2.591	F(1,36)= 1.093
	spikes/burst	F(1,36) =4.931*	F(1,36)= 2.996 p=0.09	F(1,36)= 1.608
	intraevent interval	F(1,36) =0.277	F(1,36)= 0.321	F(1,36) = 0.506
	interevent interval	F(1,38) =8.814**	F(1,38)= 3.711 p=0.06	F(1,38)= 5.926*
♂ CON vs ♂ PNA	frequency	F(1,34) =8.368**	F(1,34)= 0.7651	F(1,34)= 1.832
	duration	F(1,31) =4.331*	F(1,31)= 0.6023	F(1,31)= 0.9594
	spikes/burst	F(1,31) =5.874*	F(1,31)= 0.05163	F(1,31)= 0.502
	intraevent interval	F(1,31) =0.9099	F(1,31)= 0.9165	F(1,31)= 0.2845
	interevent interval	F(1,33) = 0.8984	F(1,33)= 1.751	F(1,33)= 0.2273

A, adult; CON, control; *p < 0.05; **p < 0.01; ***p < 0.001

Discussion

PCOS is the most common infertility disorder in reproductive-aged women. While the underlying causes are still emerging, studies suggest that in addition to genetic associations (39), high levels of circulating androgens and altered placental steroidogenesis in pregnant women with PCOS could expose the fetus to an altered

endocrine milieu (50,86). We and others have used prenatal exposure to androgens (PNA) to construct animal models that recapitulate many aspects of PCOS, enabling potential underlying mechanisms to be studied (51-54,58,87). Based on growing clinical evidence that altered neuroendocrine activity manifests in hyperandrogenemic girls during the pubertal transition (70,71,88,101), we used the PNA mouse model to examine GnRH neuron activity during development and whether or not this is altered by PNA and/or is sexually differentiated. GnRH neurons are remarkably active in prepubertal mice, and their activity is affected by PNA treatment, sex and age. These results suggest GnRH neurons are not merely 'turned on' at the pubertal transition, but play a role throughout development; disruption of this early-life activity may contribute to PCOS.

Striking sex differences were observed in the development of GnRH neuron firing activity. Firing rate of GnRH neurons from control females increased steadily through the first three weeks of development. In contrast, male firing activity decreased from one to two weeks of age. This result is consistent with the previously observed decline in GnRH release from one to two weeks of age in brain slices from male mice (78). The pattern of GnRH release during prepubertal development in females has not been characterized, but the observation that release at the median eminence is largely dependent upon action potentials (102) suggests release may follow a pattern similar to firing in females, and thus be different than males. It is notable that despite these sex differences in GnRH neuron activity during development, females and males share the surprising finding that activity of GnRH neurons peaks well before any outward signs of puberty are evident.

Because PNA increases reproductive neuroendocrine output in adults in all species studied, we postulated GnRH neuron firing rate would be greater in PNA than control females during the prepubertal period. Contrary to this hypothesis, PNA exposure reduced GnRH neuron activity during prepubertal development, most notably at three weeks of age, although the decline appears to manifest earlier. This result is of interest with regard to studies examining pulsatile LH release in young obese girls. LH pulse frequency is reduced in these girls compared to controls at in early Tanner stages (Tanner 1-2). This was reversed, however, in later stages (Tanner 4-5) with LH pulse frequency being higher than controls (103). Circulating androgens increase with BMI quintile (69), thus obese girls have a higher tendency to be hyperandrogenemic, although the small numbers in the above study precluded a statistical identification of hyperandrogenemia (103). No animal model can perfectly recapitulate a human disease, but the present data showing an initial suppression of GnRH neuron activity during development coupled with elevated GnRH neuron activity (63) and increased LH-pulse frequency in adult PNA females (59,63) seems to parallel these clinical findings. Of note, no difference was detected in firing rate of GnRH neurons from adult PNA vs control mice in the present study; we attribute this to the loss of statistical power with the high number of comparisons made. The means are similar to the previous report and a two-way test of adult values achieves significance.

In contrast to females, PNA had no effect on firing rate in cells from male mice. This supports and extends previous work showing that few neuroendocrine deficits causing fertility problems have been observed in male offspring of women with PCOS (104). These offspring do exhibit metabolic deficits and higher anti-Müllerian hormone levels

(37,104), but these are downstream and/or tangentially related to the reproductive neuroendocrine axis. Whether or not PNA treatment in females merely masculinizes the GnRH neurosecretory system is an obvious question, given androgens are typically elevated in male fetuses during development to facilitate differentiation of the genitalia in a male pattern (105). In the present studies, the suppression of overall firing rate before puberty in PNA compared to control females suggests masculinization. Differences between PNA females and control males were observed, however, in action potential firing patterns. In controls from both sexes, burst frequency declined with age. Interspike interval also shifted to longer intervals with age and became more irregular. While burst properties were not affected by PNA, the interval distribution in adult PNA females was different than control males. This difference in action potential patterning argues against masculinization as a simple explanation for effects of PNA and suggests finer details of GnRH neuron firing may be altered by PNA exposure.

The observation that GnRH neuron firing rate peaks before puberty indicates that the main role of GnRH neuron activity before activation of the downstream reproductive system may be to unrelated to reproduction *per se*. In adults, GnRH is released not only in the median eminence, but also from the perisomatic region and proximal processes (102,106). The former regulates pituitary gonadotropin output but the latter acts as a neuromodulator to alter central circuits, including regulating GABAergic transmission to GnRH neurons (107). High-frequency depolarization of GnRH neurons can also affect short-term plasticity in GABAergic transmission to these cells via glia and/or endocannabinoid intermediates (108). Neuronal activity plays many roles in the development of neuronal circuits, including formation, maturation, and refinement of

synapses (79,109,110). Synapse formation is driven by a competitive process involving strong activity input from the postsynaptic cell (111,112). The present observations of higher burst frequency in GnRH neurons from younger animals are consistent with this. Classic studies in the cat visual cortex demonstrated that blocking activity leads to reorganization of synaptic connections and functional output of retinal ganglion cells (113). Decreasing activity of cultured hippocampal neurons before synapse formation decreased functional inputs to that cell. In contrast, decreasing activity after formation of synaptic connections increases the number of synapses to the cell as a way to restore activity (114).

An alternative explanation for the PNA-induced decline in GnRH neuron activity during development is that changes in GnRH neuron activity reflect postsynaptic changes initiated within the GnRH neuron to compensate for altered synaptic input. In this regard, a preliminary report indicates prepubertal GABAergic transmission to GnRH neurons is increased at three weeks of age in PNA mice (115), corresponding to the time when PNA reduced GnRH firing rate in the present study. GABAergic transmission is typically excitatory in these cells (66,116). Consistent with the observed increase in GABAergic transmission, appositions from GABAergic processes that originate in the arcuate nucleus of the hypothalamus are increased in PNA mice as early as postnatal day 25 (117). Whether changes in GnRH neuron activity or the presynaptic network come first is unknown. Both pre and postsynaptic components play a role in the final maturation and establishment of a synapse (118), supporting the idea that either altered activity and/or GABAergic inputs in PNA animals may affect synaptogenesis within the GnRH network. GABA can act as a paracrine factor to help establish synapses

(119,120). Activity of the postsynaptic cell can also modify postsynaptic scaffolding proteins that stabilize receptors (121). Additionally, both activity and GABA are thought to play a role in the retraction of synapses. Reducing GABA release in basket interneurons by knockdown of the vesicular loading transporters decreases branch pruning and increases the number of synaptic boutons (122), and postnatal activity in the visual cortex drives the engulfment of synapses via microglia (123). Together, these observations suggest the postulate that GnRH neuron activity before puberty is important for attracting, pruning and refining synaptic connections in the postnatal GnRH network; the suppression of activity in PNA females is therefore likely to alter these processes.

Prenatal exposure to androgens may act centrally to program changes in the circuitry and/or activity of GnRH neurons. GnRH neurons, however, do not express detectable levels of the androgen receptor (124), suggesting androgens initiate the cascade of events that program PNA-induced changes in another cell type. Androgen receptor expression in the early embryonic brain has not been well characterized. In rats, mRNA has been detected at E20 in multiple brain regions (125), whereas immunohistochemical studies in the mouse hypothalamus detected androgen receptors at P5 but not E19 (126). Neither of those studies examined androgen receptor within GABAergic neurons, however this receptor is expressed in GABAergic cell populations of the adult hypothalamus (127). Another possibility is that PNA alters other processes that then affect neural circuits, for example postnatal androgen production. Hyperandrogenemic women with PCOS have mild elevations of androgen, lower than typical male levels. In primates, exposure to a similar mild elevation in testosterone

during the prepubertal period increases LH pulse frequency around puberty (74). Of interest in this regard, androgens increases GnRH neuron firing in brain slices from adult mice of both sexes (29,57) and also up regulate GABA transmission to adult GnRH neurons in an activity-dependent manner (28). Programming and activational effects of androgens in this system are not mutually exclusive and both may be contributing to the PNA phenotype during development and in adulthood. Of note, it is not known when changes in androgen levels occur in PNA mice during development, thus whether or not the observed developmental changes are solely attributable to prenatal androgen exposure cannot be determined from these studies.

The present demonstration that final common pathway of the reproductive neuroendocrine axis is active prior to puberty, is altered by PNA and is sexually differentiated, provides insight into both typical and pathological prepubertal development. If GnRH activity during this early postnatal developmental stage does indeed sculpt the afferent network, sex differences in activity may be critical for attraction of inputs needed for sexually-differentiated reproductive functions, such as female-specific generation of a GnRH surge in most mammals. The early suppression of GnRH neuron activity by PNA in females may produce changes that ultimately lead to upregulation of reproductive neuroendocrine axis observed in many adult PNA models and women with PCOS. An interesting, albeit speculative, possibility raised by these observations is that changes in neural activity could be the initial insult in a cascade that, in some cases, independently leads to PCOS, perhaps particularly in individuals in which one of several gene associations that have now been made are not identifiable (39,41,128,129). Together these findings suggest the postulate that GnRH neurons

have a typical neuronal role, setting up the neural network needed for later reproductive function, before they assume the neuroendocrine role regulating fertility with which they are traditionally associated.

Chapter 3 Ovarian androgens maintain high gonadotropin-releasing hormone neuron firing rate in adult prenatally androgenized female mice

Co-authored with Suzanne M. Moenter

Abstract

Gonadotropin-releasing hormone (GnRH) neurons drive reproductive cycles by varying hormone release frequency. Polycystic ovary syndrome (PCOS) is characterized by persistent high frequency GnRH/LH pulses and hyperandrogenemia. Adult prenatally androgenized (PNA) mice exhibit similar changes. In prepubertal PNA mice, however, GnRH neuron action potential firing rate is reduced. The mechanisms underlying firing rate differences in PNA mice and the age-dependent shift are incompletely understood. In nascent PCOS and in PNA mice, reproductive disruptions occur peri-pubertally, when gonadal steroid production increases. We hypothesized dysregulated ovarian feedback increases GnRH neuron activity in adult PNA mice, but not before puberty. To test this, we recorded GnRH neuron firing rate 5-7 days after sham-operation, ovariectomy (OVX), or OVX plus an implant producing sub-male levels of androgens (OVX+DHT). In adult control mice, OVX increased and OVX+DHT further increased GnRH neuron firing rate. In marked contrast, OVX decreased firing rate in adult PNA mice and OVX+DHT restored firing rate to sham-operated levels. In contrast, there was no effect of OVX (14-16d, record 3wks of age) on GnRH neuron activity before puberty in PNA mice, although sham surgery itself appears to reduce firing activity. Ovarian androgens were

thus measured and were not different between groups at three weeks of age. These results suggest prenatal and postnatal androgens play different roles. Prenatal androgens program not only low GnRH neuron activity before puberty, but also dependence on ongoing androgen stimulation to maintain activity in adults. Post-pubertally, mild elevation of androgens activates GnRH neurons in both control and PNA mice.

Introduction

GnRH neurons are a crucial component of the axis governing reproduction. GnRH stimulates release of luteinizing hormone (LH) and follicle-stimulating hormone by the pituitary; these hormones activate gonadal functions including steroidogenesis. Gonadal steroids exert feedback control of GnRH neuron firing rate and pulsatile release. Proper patterns of pulsatile GnRH release are required for fertility (8,80,82,83), thus it is not surprising that atypical GnRH/LH pulse frequency underlies several reproductive disorders, including polycystic ovary syndrome (PCOS) (82). PCOS is a common multifaceted condition characterized by disrupted cycles and, often, hyperandrogenemia. It is estimated that as many as ten percent of reproductive-age women have PCOS by classic NIH criteria, and up to twenty percent by the currently used Rotterdam criteria (19). In PCOS, persistent high-frequency LH, and presumably GnRH, pulses increase androgen levels and thereby alter steroid feedback (84,85). Some of these changes occur around the time of puberty (71,130) but the mechanisms driving persistently high-frequency GnRH/LH pulses are not fully understood.

It is not possible to study the central neurobiological mechanisms that may contribute to altered GnRH/LH pulse frequency in PCOS patients, thus prenatally androgenized animal models, which mirror many aspects of PCOS, have been used (131,132). Similar to women with PCOS, PNA females exhibit increased LH pulse frequency, disrupted reproductive cyclicity, and elevated androgen levels (51-54,87). In adult PNA mice, GnRH neuron firing rate is elevated, likely in part driven by increased excitatory GABAergic input (58,59,63). Interestingly, before puberty (measured by vaginal opening) GnRH firing rate is lower in PNA females (133) despite elevated GABAergic input that remains depolarizing (134). It is unclear if changes in GnRH neuron firing rate either prepubertally or in adults are driven though altered organizational programming of neuronal mechanisms, and/or if factors from the ovary, in particular androgens, play an activating role.

In the present study, we tested the influence of ovarian factors on GnRH neuron firing rate in control and PNA mice at three weeks of age and in adulthood. Our hypotheses were 1) ovarian factors contribute to increased firing in adult PNA animals, 2) androgens are the bioactive ovarian factor contributing to this increase, and 3) ovarian factors do not impact GnRH neuron firing rate in prepubertal animals.

Methods

All chemicals were acquired from Sigma Chemical Company (St. Louis, MO, USA) unless noted.

Animals: Mice expressing GFP under a control of the GnRH promoter on a C57Bl6/J background (GnRH-GFP mice) were given *ad libitum* access to water and chow (Teklad

2916, except breeders which received the higher protein 2919 chow) (Envigo, Madison Wisconsin, USA). Mice were housed on a 14L:10D light cycle with time of light on at 0300 Eastern Standard Time. PNA mice were generated as described (58,133). In brief, adult female mice were injected with 225 μg DHT in sesame oil on d16-18 of gestation (d1=copulatory plug). Control groups included mice injected with sesame oil vehicle and uninjected dams. No difference was observed between offspring of control and vehicle-treated animals and these are combined and reported as controls. In all breeding cages, a CD1 mouse was included for maternal and nutritional support to increase survival of PNA pups; combined litters were adjusted to <15 pups by culling CD1 pups, which are phenotypically distinct, to normalize nutrition. The Institutional Animal Care and Use Committee of the University of Michigan (PRO00006816/PRO00008797) approved all animal procedures.

To study the effects of ovarian factors on GnRH neuron action potential firing rate, mice underwent ovariectomy (OVX) or sham surgery, in which similar incisions were made and the ovary externalized. Surgery or sham was performed under isoflurane anesthesia with bupivacaine applied post operatively as a local analgesic. To study if androgens alter firing rate, a Silastic (Dow Corning, Midland, MI, USA) capsule containing 400 μg of DHT in sesame oil was implanted in the scapular region of some mice during ovariectomy (OVX+DHT). Recordings were made 5-7 days following surgery. Sham-operated mice were in diestrus (determined by vaginal cytology and control uterine mass >75 mg) on the day of recording.

Slice preparation. Solutions were bubbled with 95% O_2/5% CO_2 throughout the duration of experiments and at least 15 minutes prior to tissue exposure to solutions. Slices were

made between 07:45-14:00 EST. The brain was rapidly removed and placed in ice-cold sucrose saline solution containing (in mM): 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 NaH_2PO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal (300 μm) slices were cut with a Leica VT1200S (Leica Biosystems, Buffalo Grove, IL, USA). Slices were incubated in a 1:1 mixture of sucrose saline and artificial cerebrospinal fluid (ACSF) containing (in mM): 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO4, 1.2 $MgSO_4$, 2.5 $CaCl_2$ (pH 7.4) for 30 min at room temperature (21- 23 ºC) and then transferred to 100% ACSF for additional 30 min at room temperature before recording. Recordings were performed 1-5 h after brain slice preparation; no difference in firing patterns were evident based on time after brain slice preparation or time of day.

Extracellular recordings. Extracellular recordings, which have minimal impact on the cell, were made to monitor spontaneous action potential firing (91,92,133) . Slices were placed in a chamber continuously perfused with ACSF (2-3ml/min) and heated to 30-32ºC with an inline-heating unit (Warner Instruments, Hamden, CT, USA). To identify GnRH neurons, an Olympus (Center Valley, PA, USA) BX51WI microscope was used to briefly illuminate GFP-positive cells in the preoptic area at 488 nm. A Flaming/Brown P-97 puller (Sutter Instruments, Novato, CA USA) was used to pull borosilicate capillary glass (type 7052, 1.65 mm outer diameter; 1.12 mm inner diameter; World Precision Instruments, Inc. Sarasota, FL, USA) into recording micropipettes with a resistance of 2-4 MΩ. Micropipettes used for recordings were filled with HEPES-buffered solution containing (in mM): 150 NaCl, 10 HEPES, 10 glucose, 2.5 CaCl2, 1.3 MgCl2, and 3.5 KCl. All recordings were conducted on one channel of an EPC-10 dual patch clamp amplifier using Patchmaster software (HEKA Elektronik, Pfalz, Germany) running on a

Macintosh computer. Low resistance seals (<28MΩ) were formed between the pipette and neuron after first exposing the pipette to the slice tissue in the absence of positive pressure. Recordings were made in voltage-clamp mode with a 0 mV pipette holding potential and signals acquired at 10 kHz and filtered at 5 or 10 kHz. Recording stability and loose seal were checked every 10 minutes by current response to a 5mV hyperpolarizing voltage step. At the end of each experiment, inactive cells were treated with high-potassium ACSF (20 mM K^+). Cells that exhibited action currents in response were verified to be alive and recordable, and all data were used. For cells not responding to K^+, data analysis was truncated at the last observed action current.

Experimental design Brain slices from three-week-old and adult (15-31 wks of age) female control and PNA mice that had been sham-operated or OVX 5-7 day previously were used. Three-week-old mice were not weaned before experiments to avoid abrupt changes in nutrition and separation stress. An additional group of unoperated three-week-old control mice was tested to determine the effect of sham surgery. One-hour recordings of GFP-identified GnRH neurons were made. No more than three cells per animal and two cells per slice were included for analysis, and at least four mice from at least three different litters were tested per group. Variation within an animal or among littermates was not less than among all animals within a group. Cell location was mapped to an atlas (135) after recording; no differences in recording parameters were attributable to location.

LH assay Serum (6µl) was diluted in 54µL of 0.1M PBS with 0.05% Tween 20. Samples were stored at −20 °C until LH assay based on a method by Steyn et al. (136). The capture monoclonal antibody (anti-bovine LH beta subunit, 518B7) is provided by Janet

Roser, University of California. The detection polyclonal antibody (rabbit LH antiserum, AFP240580Rb) is provided by the National Hormone and Peptide Program (NHPP). HRP-conjugated polyclonal antibody (goat anti-rabbit) is purchased from DakoCytomation (Glostrup, Denmark; D048701-2). Mouse LH reference prep (AFP5306A; NHPP) is used as the assay standard. The Limit of Quantitation (functional sensitivity) was 0.016 ng/ml, defined as the lowest concentration that demonstrates accuracy within 20% of expected values. Intraassay coefficient of variation (%CV) was 2.2% determined by serial dilutions of a defined sample pool.

Analysis Action currents (events) were detected off-line using custom programs in Igor Pro 6.31 and 7.02 (Wavemetrics, Lake Oswego, OR, USA). Data were binned at 60-sec intervals and were transferred to Excel. Mean firing rate (Hz) was calculated by dividing the total number of events by the duration of recording. Short-term firing patterns (bursts), which can affect neurosecretion (65), were also compared. Events were considered to be part of a burst if they occurred within 0.36s of the preceding event (133). Burst frequency, duration and number of spikes/burst were compared among groups.

Statistics. Statistical analyses were performed using Prism 7 (GraphPad Software, La Jolla, CA, USA). Data are reported as individual values with mean±SEM. Data distributions were tested using Shapiro-Wilk. Adult data were not normally distributed and were log transformed before statistical analysis. Two-way ANOVA with Fisher's LSD *post hoc* test was used to compare groups for both adult and three-week-old data. Mann-Whitney U-test was used to compare firing rates between unoperated and sham-

operated control mice at 3-weeks of age. The null hypothesis was rejected if $p<0.05$, but all p values <0.1 are reported.

Results

Ovariectomy increases GnRH neuron firing rate in adult control but decreases firing rate in adult PNA mice. Ovarian feedback to neuroendocrine reproductive circuitry matures during the pubertal process. We tested if ovarian factors contribute to the increased firing rate in PNA adult mice by making extracellular recordings of action potential firing rate of GnRH neurons from sham-operated and OVX mice. Representative traces are shown in Figure 3-1 and summary data in Figure 3-2A. Cells from sham-operated PNA mice had a higher firing rate than cells from sham-operated controls as reported for unoperated mice (63,133). In control mice, OVX increased firing rate, consistent with removal of negative feedback ($p<0.04$, sham 9 cells from 7 mice, OVX 10 cells from 7 mice). In marked contrast, OVX reduced firing rate of GnRH neurons from PNA mice ($p<0.01$, sham-PNA 12 cells from 7 mice, PNA-OVX 13 cells from 8 mice). This observation suggests that signals from the ovary drive increased GnRH neuron firing rate in PNA mice, and also points to dysfunctional feedback in these mice.

Mild hyperandrogenemia is common in women with PCOS and likely is a factor contributing to increased GnRH/LH pulse frequency (21). We thus hypothesized that replacement of androgen would increase GnRH neuron firing rate in control mice, and would restore firing rate in PNA mice. To test this, we used DHT implants that provide a level of androgen exposure that does not restore seminal vesicle mass in castrate males, thus is lower than in typical males (57); this is done to mimic the mild androgen elevation observed in women with PCOS. Both of the above hypotheses were

supported. GnRH neurons from OVX+DHT control mice had increased firing rate relative to sham-operated controls and approached the level set for significance for an increase when compared to OVX controls ($p=0.06$, 12 cells from 7mice). In cells from OVX+DHT PNA mice, firing rates were elevated relative to OVX PNA ($p<0.01$, 11 cells from 6 mice) and were not different those in sham-operated PNA mice. Together these data suggest that androgens are the primary factor from the ovary contributing to increased firing in PNA mice, and that androgens can also elevate firing in control mice.

Short-term firing patterns are altered by gonadal state in both control and PNA mice. Short-term firing activity, also known as bursting activity, is related to peptide neurosecretion (65,137), an aspect postulated to be increased in both women with PCOS and PNA in models. Analysis of short-term firing patterns or “bursts” in control mice demonstrated that OVX alone did not alter burst frequency ($p>0.73$). In cells from OVX+DHT mice, however, burst frequency (Figure 3-2B) was increased compared to cells from both sham-operated and OVX control mice ($p<0.03$). Burst frequency was increased in cells from PNA-sham compared to control-sham mice ($p<0.04$). Consistent with the decline in firing rate, burst frequency was lower in cells from PNA-OVX than control-OVX or PNA-sham mice ($p<0.007$). In cells from OVX+DHT PNA mice, burst frequency was increased relative to cells from PNA-OVX mice ($p<0.0003$) and not different from cells from PNA sham mice ($p>0.29$). Burst duration (Figure 3-2C) was elevated in cells from sham-PNA vs sham-control mice ($p<0.01$). In control mice, burst duration increased with both OVX ($p<0.03$) and OVX+DHT ($p<0.002$) compared to sham operated controls. In contrast, within PNA mice, no treatment affected burst

duration (all p>0.31). No treatment altered the number of spikes per burst (Figure 3-2D, all p>0.15).

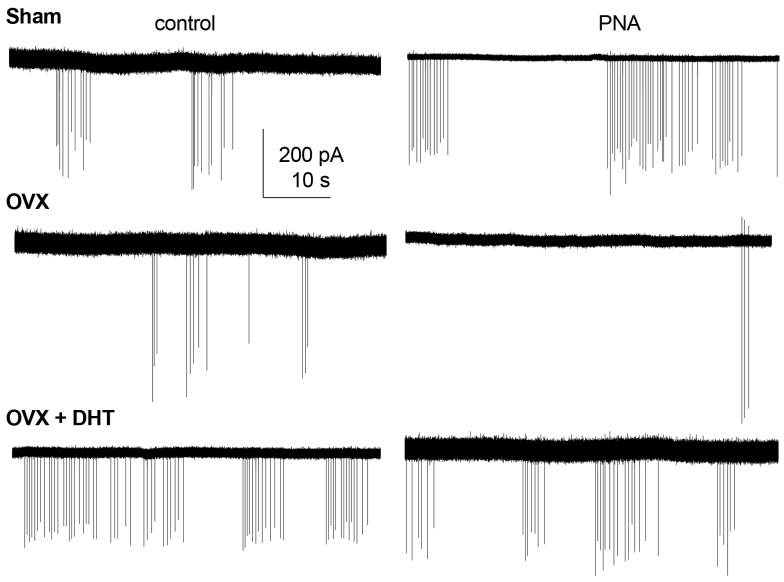

Figure 3-1. Gonadal status and androgens affect long-term firing rate of GnRH neurons from both control (left) and PNA (right) adult mice. Representative traces from sham-operated (top), OVX (middle) and OVX+DHT (bottom).

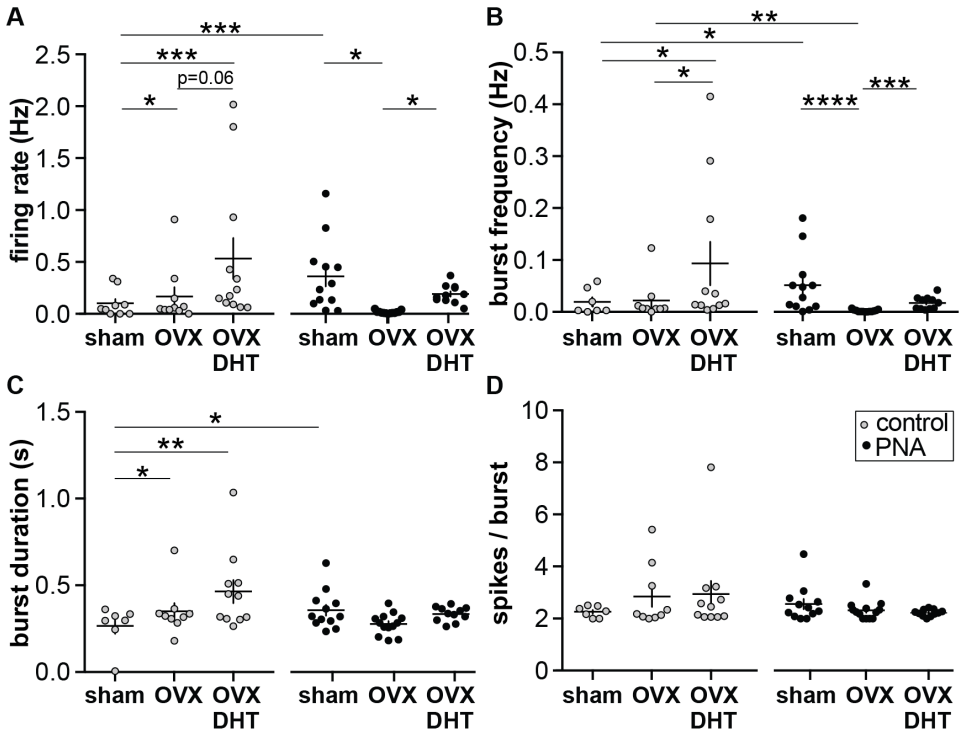

Figure 3-2. Summary of effect of gonadal status and androgens on long-term and short-term (burst) firing properties of GnRH neurons. Individual values and mean±SEM for (A)

firing rate, (B) burst frequency, (C) spikes/burst, and (D) burst duration. * p<0.05, ** p<XXX, *** p<0.001, **** p<0.0001, two-way ANOVA, Fisher's LSD.

Ovariectomy does not alter firing rate in three-week-old PNA mice. To test if gonadal inputs influence firing rate in control and PNA mice before puberty, extracellular recordings were made from GnRH neurons of OVX and sham-operated mice at three weeks of age. Ovariectomy and sham surgeries were conducted on postnatal day 14-16 (5-7 d prior to recording). Representative recordings are shown in Figure 3-3A and mean firing activity and burst analysis for individual cells are plotted in Figure 3-3B-E. Ovariectomy did not affect firing rate in either group (control sham, 16 cells from 8 mice, vs control-OVX,14 cells from 7 mice, p=0.74, and PNA-sham,12 cells from 6 mice, vs PNA-OVX, 11 cells from 6 mice, p= 0.51). All burst parameters did not appear different between any of these groups (p>0.1, all comparisons for SPB, MBD and BF). Notably, firing rate of cells from sham-operated three-week-old control mice was lower than reported (133) and similar to those from three-week-old PNA mice in the present study (p=0.79). We thus hypothesized that surgery at such a young age has an inhibitory influence on GnRH neuron firing rate that persists to at least three weeks of age. To test this, recordings were made from unoperated three-week-old control mice. Preliminary results (Figure 3-3F support this hypothesis and indicate that surgery may confound our results. Specifically, with the unoperated group half completed, there is trend for increased firing compared to the sham group (one-tailed Mann-Whitney test, p=0.07, n=8 unoperated vs n=16 sham).

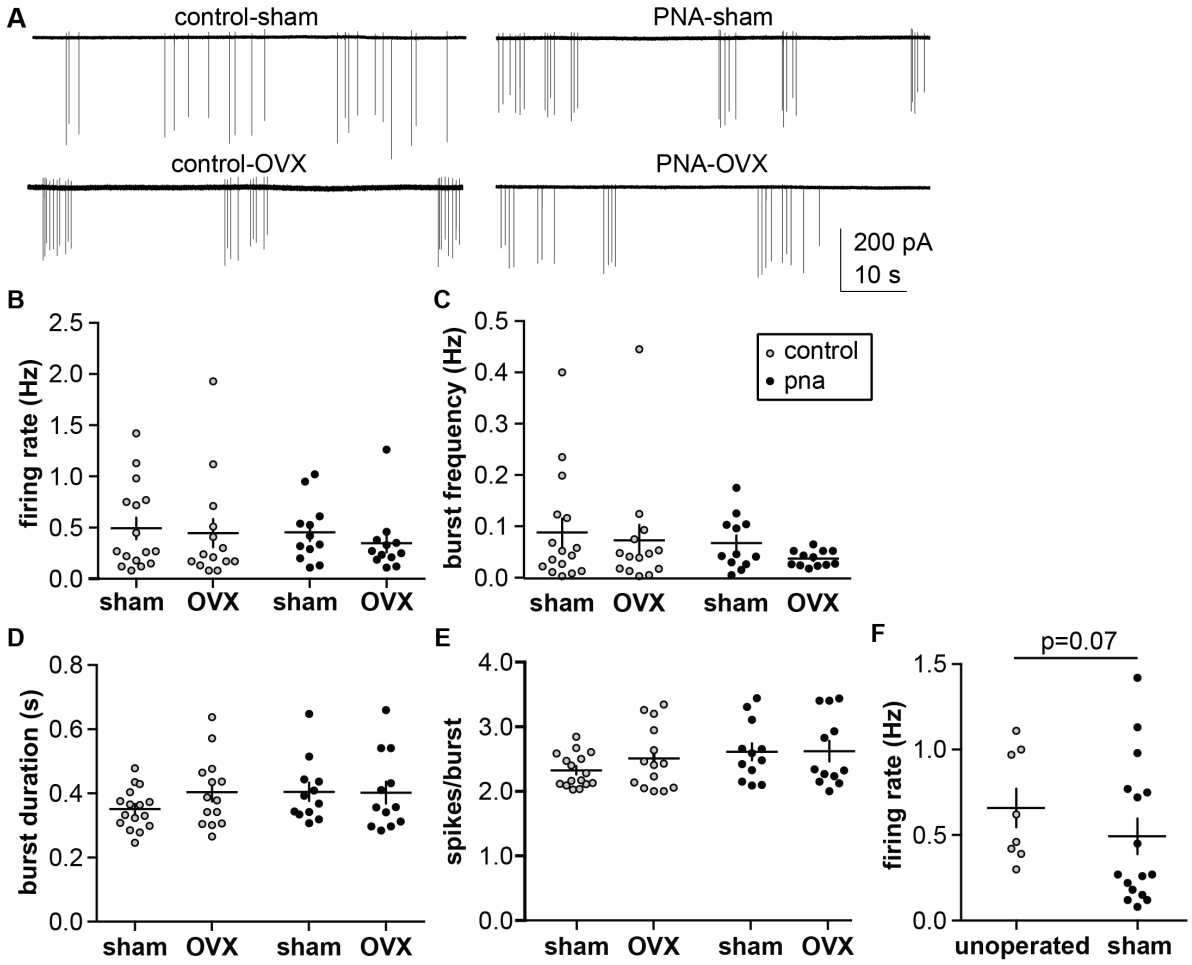

Figure 3-3. Gonadal status does not alter and postnatal surgery may suppress firing rate of GnRH neurons in three week old mice. A. Representative traces from control (left) and PNA (right), with sham operated on top and OVX on bottom. B-E. Individual values and mean±SEM for (B) firing rate, (C) burst frequency, (D) spikes/burst, and (E) burst duration. F. Firing rate in unoperated mice compared with cells from the above sham operated controls. * p<0.05, ** p<0.005, *** p<0.001, **** p<0.0001, two-way ANOVA, Fisher's LSD for B-E, one-tailed Mann-Whitney test for F.

Serum LH response differed from GnRH activity response. Serum LH was assessed in trunk blood collected at the time of brain slice preparation (Figure 3-4). In control mice, OVX increased LH levels (p<0.001, two-way ANOVA/Fishers). Surprisingly, PNA mice exhibited a similar post-ovariectomy rise (p<0.001, two-way ANOVA/Fishers). DHT replacement did not alter LH levels from those in OVX mice in either control or PNA mice.

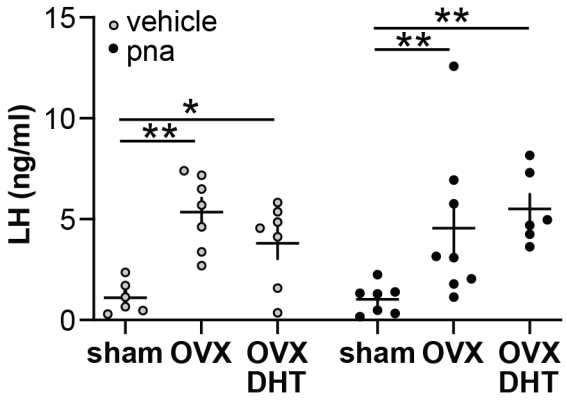

Figure 3-4. Serum LH values from adult mice used for extracellular recordings. *p<0.05, **p<0.01 two-way ANOVA, Fisher's LSD; interaction F(2,35)=1.1, p=0.3286; steroid F(2,35)=13, p<0.0001; vehicle vs pna F(2,35)=0.16, p=0.6906.

Discussion

Hyperandrogenemia is a common facet in many women with PCOS, as well as in several animal models used for studying this disorder. Here we demonstrate that prenatal androgen exposure has marked effects on the typical homeostatic operation of the hypothalamo-pituitary-ovarian axis. Ovarian androgens in adult PNA mice strongly influence the firing rate of GnRH neurons, but this influence is absent before the pubertal transition. Remarkably, the regulation by the ovaries of GnRH neuron firing rate and LH appear to be opposite in adult PNA mice, suggesting potential compensatory mechanisms at the pituitary or other central circuits.

These studies support previous work that demonstrated elevated androgens can upregulate adult reproductive neuroendocrine output in PCOS patients (82) and in adult PNA models (58,61). Mild androgen elevations occurring naturally in women with PCOS (138) disrupt the efficacy of progesterone negative feedback to reduce LH pulse frequency (27). Mild androgen increases achieved in animal models has similar activational effects (58,74,139). For example, impaired progesterone negative feedback

has also been observed in PNA sheep (140) and rats (53), suggesting early exposure to androgens can disrupt later ovarian feedback. In young rhesus monkeys, mild androgen elevation increases LH pulse frequency (74); similarly, sub-male levels of androgen in combination with estradiol increase GnRH neuron firing rate in mice (29) and increase excitatory GABAergic drive to GnRH neurons (28). Postnatal treatment of rodents with the aromatase inhibitor letrozole also elevates androgens and LH levels, and reduces progesterone receptor mRNA levels in the hypothalamus (141). Prenatal anti-Müllerian hormone (AMH) models were developed based on of observations that AMH is increased in women with PCOS during the second trimester. Injection of AMH during pregnancy on the same days as DHT is injected to produce PNA mice produces prenatal androgen elevation. Perhaps not surprisingly, female offspring from prenatal AMH mice display similarly disrupted cycles and increased LH pulse frequency and elevated testosterone (142). In both prenatal AMH and PNA mice GABAergic input sites and spine density of GnRH neurons are increased, as is GABAergic transmission to GnRH neurons in the latter (58,59,142). Mild androgen elevation is the common denominator among these diverse models and is consistently associated with increased reproductive neuroendocrine activity.

The present work extends the above findings by demonstrating that PNA treatment programs changes in the reproductive neuroendocrine system that markedly disrupt the typical operation of homoeostatic feedback at the central level. Specifically, in control mice ovariectomy resulted in the expected increase in GnRH neuron firing rate due to removal of ovarian negative feedback. In marked contrast, in ovariectomized PNA mice, GnRH neuron firing rate plummeted. This suggests the postulate that PNA treatment

rewires the system so that GnRH neuron function in the adults becomes dependent upon ongoing androgen exposure. This postulate is supported at least in part by the restoration of firing rate in ovariectomized PNA mice treated with DHT. Of interest, DHT also tended to increase firing rate in control mice. Together present and past findings strongly indicate that mildly increased androgens in females upregulates reproductive neuroendocrine output, and suggest similar changes may underlie phenotypes commonly observed in PCOS. These studies also compliment clinical studies in PCOS patients that have identified a role for the ovary in deficits found in PCOS. For example ovarian surgeries such as wedge resection, ovarian drilling and electrocautery have been used as a method to improve fertility outcomes in PCOS patients. In the majority of studies such surgeries were found to lower LH and testosterone levels (143), however this did not occur in all cases (144).

Examination of LH levels in these same mice on the day brain slices were prepared revealed a surprising differential regulation of the hypothalamus and pituitary gland. In control mice, the expected elevation of LH was observed following ovariectomy. In striking contrast to the inhibitory effects of removing the ovaries on GnRH neuron firing rate in PNA adults, LH levels rose after ovariectomy just as in controls. There are several possible explanations for this. First, negative feedback actions of other ovarian steroids may be more pronounced at the pituitary than the hypothalamus in PNA mice. This is consistent with the above-mentioned studies demonstrating that elevated androgens disrupt negative feedback at the hypothalamus. Second, pituitary response to GnRH may be increased in PNA mice as it is in other PNA models and in women with PCOS (52,53,87,145). Third, the relationship between GnRH neuron firing activity and

GnRH release could be altered by PNA, so that relatively little firing activity produces increased release. Fourth, PNA and/or ovariectomy may alter substances, such as gonadotropin-inhibitory hormone, that can alter LH release independent of GnRH (146,147).

Of interest, the activational effects of ovarian androgens were confined to adults, as ovariectomy had no effect at three weeks of age. We urge caution in the interpretation of these results as sham surgery appears to have an inhibitory effect on GnRH neuron firing rate at this young age. We have in progress studies to measure the ovarian androgen content at three weeks of age. Androgen levels in the circulation of even adult female mice are typically below the level of functional sensitivity in reliable immunoassays. Because of their lipophilic nature, androgens cannot be stored for regulated secretion, thus ovarian levels should be correlated with serum levels (148-150). If ovarian androgen levels are, as expected, low, this will suggest there is little ovarian drive to the system before steroidogenesis matures. If ovarian androgens further do not differ between control and PNA mice, this will add weight to the potentially confounded measure of a lack of effect of ovariectomy on GnRH neuron activity in prepubertal mice. Other mechanisms, such as altered androgen receptor expression or signaling, could still produce changes, thus these studies are viewed as an early indication of a minimal effect of ovarian androgens on reproductive neuroendocrine output before puberty.

In addition to overall mean firing rate, short-term firing changes have implications for hormone release. For example, the duration and number of spikes in a burst is often correlated with a greater neuropeptide release (98). In the present study, changes in

burst firing largely paralleled those in overall firing rate, adding further support to androgen alteration of neuroendocrine output. In cells from control mice, burst duration was shorter for sham-operated mice than for OVX or OVX+DHT. Sham-operated PNA mice also exhibited longer bursts than sham-operated controls. Together these results suggest that androgens in female mice increases burst length. This is in contrast to a recent study in male mice in which orchidectomy, which would reduce androgens, increased burst length in arcuate kisspeptin neurons (151). Increased burst length would potentially generate a longer elevation in intracellular calcium and thus greater neuropeptide release (98). In males, orchidectomy removes a higher level of androgen that produces negative feedback on arcuate kisspeptin neurons (151), but interestingly not GnRH neurons (57). In females, a mild elevation of androgen via PNA or via DHT replacement increases burst duration and thus potentially hormone release.

Disrupted neuroendocrine function and hyperandrogenemia emerge near the pubertal transition and may contribute the foundations of increased reproductive neuroendocrine drive in adult PCOS. While no animal model can perfectly replicate human disease, the present studies suggest that prenatal androgen exposure has programming (organizational) effects in the perinatal period, ovarian androgens have activational effects in adults, and the programming affects how the system responds to androgens and their withdrawal in adulthood (Figure 3-5).

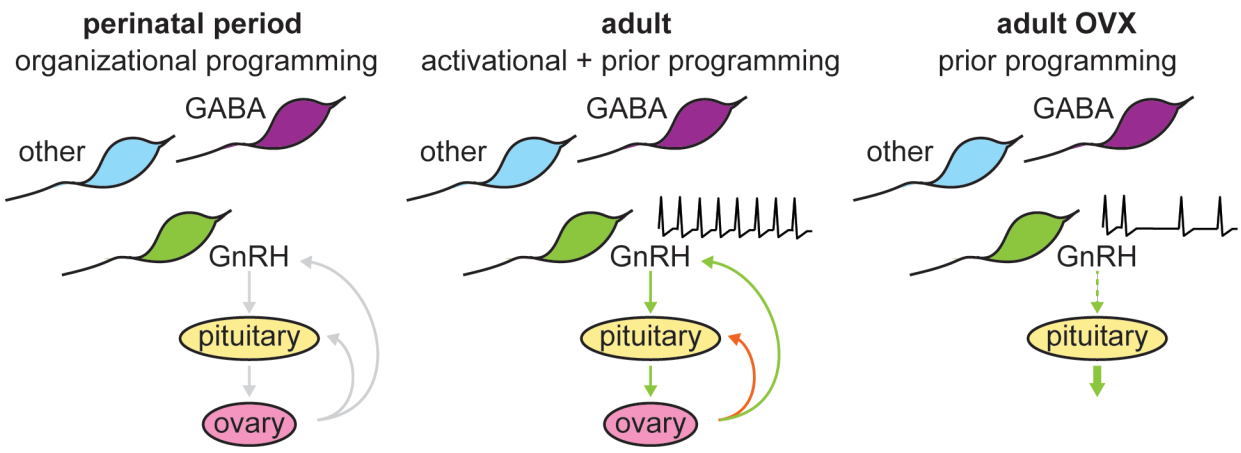

Figure 3-5. Model depicting possible organizational and activational effects of androgens. Left, prenatal androgen exposure induces organizational changes on GnRH neurons, their GABA afferents, and other central elements. Ovarian androgens are low, thus activational effects are minimal. Middle, ovarian androgens feed forward to increase GnRH release. Ovarian feedback at the pituitary is negative. Right, removal of ovarian factors eliminates activational drive to GnRH neurons and removes negative feedback from the pituitary, allowing LH to rise despite reduced GnRH neuron activity.

Chapter 4 Chemogenetic manipulation of GnRH neurons during pubertal development alters adult GnRH neuron firing rate but does not alter reproductive output

Co-authored with R. Anthony DeFazio and Suzanne M. Moenter

Abstract

Gonadotropin-releasing hormone (GnRH) neurons are central mediators of the reproductive axis through their release of GnRH, which subsequently controls pituitary and gonadal function. Recent studies demonstrated GnRH neurons are active well before outward manifestations of puberty. This prepubertal activity was also altered in prenatally androgenized (PNA) mice, in which later adult reproductive function is compromised. In other brain regions, early neuron activity plays a critical role in establishing circuitry and adult function. Here we test the hypothesis that prepubertal GnRH neuron activity has a role in adult reproductive function and in activity of the GnRH neuronal network. Either activating (HM3Dq) or inhibitory (HM4Di) designer receptors exclusively activated by designer drugs (DREADDs) were targeted to GnRH neurons using cre-lox technology. Injection of the DREADD ligand clozapine n-oxide (CNO) produced the expected changes in luteinizing hormone (LH) release from the pituitary. CNO was administered between two and three weeks of age, the period when GnRH neuron activity was reduced in PNA mice. Reducing GnRH neuron activity during development altered adult GnRH neuron firing rate but not GABA transmission to these

cells. Preliminary data suggest increasing GnRH neuron activity during development may alter adult firing rate as well. Interestingly, neither treatment had effects on timing of puberty or reproductive cycles. This study supports the hypothesis that prepubertal alteration of neuronal activity can program changes in the adult neuronal network governing reproductive function, and it indicates that compensatory mechanisms can overcome these changes at the level of whole-animal reproductive measures.

Introduction

Reproduction is required for species survival and is controlled through the hypothalamic pituitary gonadal axis. GnRH neurons are the central neuronal components of this axis; they release GnRH from terminals in the median eminence to regulate luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis and secretion. LH and FSH activate gonadal functions, including steroidogenesis. Gonadal steroid feedback to both the brain and the pituitary regulates hormone release. Disruptions in this axis can lead to infertility, which is estimated to affect up to one in six couples (1,152). The leading cause of reproductive complications in women of child-bearing age is polycystic ovary syndrome (PCOS). Hyperandrogenemic PCOS affects 8-10% of women and is characterized by oligo/anovulation, mildly elevated androgens and persistent high frequency of LH, and presumably GnRH, release. Most studies of PCOS have occurred in adults, a time when reduced fertility is easily noted and diagnosis based on established criteria is possible. Increasing evidence, however, suggests aspects of PCOS emerge before and/or during the pubertal transition.

To study mechanistic underpinnings of PCOS, animal models are needed. Prenatal androgenization (PNA) recapitulates many aspects of PCOS in several species including rodents, primates and sheep. In mice, recent work demonstrated GnRH neurons are active and receive synaptic inputs well before outward signs of reproduction are present (133,134). Further, PNA treatment altered both GnRH neuron firing rate (133) and GABA transmission to these cells before puberty, as well as in adulthood (58,134). Early neuronal activity in other areas of the brain has been shown to help set up neuronal networks by attracting and pruning synaptic inputs (79,110), but the role of prepubertal GnRH neuron activity in the reproductive system is not known.

The above correlational studies pose interesting questions regarding possible programming roles of prenatal androgen exposure vs the subsequent changes in neuronal activity observed. Specifically, PNA treatment reduces GnRH neuron activity before puberty, whereas later in development, increased activity is observed, concomitant with increased GABAergic input (which remains excitatory in these cells in adults(66)). We hypothesized that decreasing GnRH neuron activity during the prepubertal period in control mice would increase GnRH neuron activity and GABAergic inputs to these cells in adulthood and disrupt reproductive cycles. We also hypothesized that increasing GnRH neuron activity during the prepubertal period in PNA mice would reduce GnRH neuron activity in adults and restore reproductive cyclicity. To test this in a manner independent of PNA treatment, chemogenetics were used to alter GnRH neuron activity before puberty, and adult neurophysiology and reproductive parameters monitored

Methods

All chemicals were acquired from Sigma Chemical Company (St. Louis, MO, USA) unless noted.

Animals

Transgenic mice (C57Bl6/J) expressing Cre under the GnRH promoter in a BAC construct, (GnRH-Cre mice) (153), were crossed onto mice expressing GFP under control of the GnRH promoter (Tg(Gnrh1-EGFP)51Sumo MGI:6158457, GnRH-GFP mice) (154) until homozygous for both GFP and CRE (GnRH-GFP/Cre mice). Both GnRH-Cre and GnRH-GFP/Cre mice were then crossed to mice expressing a floxed cassette encoding one of two versions of a DREADD (Designer Receptor Exclusively Activated by Designer Drugs), mCitrine and the HA tag (Figure 4-1). In these mice, when Cre is present a floxed stop cassette is removed and the CAG promoter drives expression of the cassette. The DREADDs utilized in these studies were hM3Dq (Dq, JAX, 026220) and hM4Di (Di, 026219), which induce the canonical G_q pathway and the canonical G_i respectively, were used. These receptors are activated by the DREADD ligand clozapine-*N*-oxide (CNO) or its metabolite clozapine (155). Table 4-1 displays the mouse genotypes used for each experiment presented in the following studies. All mice used in this study were heterozygous for GnRH-Cre, GnRH-GFP and CAG-DREADD (hM3Dq or hM4Di), unless noted.

Table 4-1: Genotypes of mice and corresponding experiments

Abbreviation	Geneotype
GnRH-Cre	GnRH-Cre$^{+/+}$ or GnRH-Cre$^{+/-}$
GnRH-GFP	GnRH-GFP$^{+/+}$ or GnRH-GFP$^{+/-}$
GnRH-Cre/GnRH-GFP	GnRH-Cre$^{+/+}$/GnRH-GFP$^{+/+}$
GnRH-4Di	GnRH-Cre$^{+/-}$/CAG-hM4Di$^{+/-}$
GnRH-3Dq	GnRH-Cre$^{+/-}$/CAG-hM3Dq$^{+/-}$
GnRH-GFP-4Di	GnRH-Cre$^{+/-}$/GnRH-GFP$^{+/-}$/CAG- hM4Di$^{+/-}$
GnRH-GFP-3Dq	GnRH-Cre$^{+/-}$/GnRH-GFP$^{+/-}$/CAG-hM3Dq$^{+/-}$

All mice were given *ad libitum* access to water and chow (Teklad 2916, breeders received higher protein 2919 chow) (Envigo, Madison Wisconsin, USA). Mice were housed on a 14L:10D light cycle with time of light on at 0300 Eastern Standard Time. PNA mice were generated as described (58,133). In brief, adult female mice were injected with 225 µg DHT in sesame oil on d16-18 of gestation (d1=copulatory plug). Control groups included mice injected with sesame oil vehicle and uninjected dams. No difference was observed between offspring of control and vehicle-treated animals and these are combined and reported as controls. In all breeding cages, a CD1 mouse was included for maternal and nutritional support to increase survival of PNA pups; combined litters were adjusted to <15 pups by culling CD1 pups, which are phenotypically distinct, to normalize nutrition. Surgery was done under isoflurane anesthesia and bupivacaine applied as a local analgesic. Some mice were ovariectomized (OVX), and some mice received subcutaneous osmotic pumps that were removed one week later. The Institutional Animal Care and Use Committee of the University of Michigan (PRO00006816/PRO00008797) approved all animal procedures.

Immunohistochemistry To assess expression of DREADD receptors in GnRH neurons GnRH-3Dq mice (n= 2) and GnRH-4Di mice (n=1) were perfused transcardially with 4% paraformaldehyde. Brains were post fixed for 4-24 hours at 4°C, then stored in 20% sucrose ± 0.01% sodium azide, for at least 12 h for cryoprotection until sectioned at 30 µm into five series on a SM2010 R freezing microtome (Leica Biosystems, Buffalo Grove, IL, USA). Sections were taken from just caudal to the olfactory bulb through the optic chiasm for use in free-floating dual immunofluorescence using standard procedures (156). The primary antibodies were rat anti-HA high affinity (1:1000, Sigma) and rabbit anti-GnRH (EL-14, 1:10,000; generous gift from Dr. Oline Ronnekleiv, Oregon Health & Science University, Portland, OR) (157). Primary antibodies were visualized with Alexa Fluor-546 conjugated goat anti-rat and Alexa Fluor 488-conjugated goat anti-rabbit (A-11081 and A-11034, respectively; Thermo Fisher Scientific, Waltham, MA), respectively. Sections were mounted on Superfrost Plus glass slides (Thermo Fisher Scientific) and coverslipped with ProLong Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific). Immunofluorescence was detected using a fluorescent Axio Imager microscope (Zeiss, Jena, Germany). The number of cells expressing GnRH, HA, or both was counted in all sections from a single series.

Tail-tip blood collection and LH measurements All mice used for blood sampling were handled for at least two weeks before experiments. Tail-tip blood collection was performed as described (158). After a small nick at the tail tip, mice were placed on a flat surface and allowed to roam freely while 6 µl of tail blood was collected, and immediately mixed with 54 µl of 0.1 M PBS containing 0.05% Tween 20 and 0.2% BSA.

Samples were kept on ice during blood collection then stored at -20°C until LH assay. Intraassay CV was 2.2% and interassay CVs were 7.3% (low quality control [QC], 0.13 ng/ml), 5.0% (medium QC, 0.8 ng/ml), and 6.5% (high QC, 2.3 ng/ml). Functional sensitivity was 0.016 ng/ml (136). In some experiments immediately following the last sample of the frequent sampling period, mice received a single injection of GnRH (150 ng/kg, IP) and blood was collected 15 min later to test pituitary LH response.

Experiments to test the bioactivity of DREADDs expressed in GnRH neurons

Experiment 1: hM4Di (Di) To study if activation of the Di DREADD targeted to GnRH neurons could decrease LH, adult female GnRH-Cre and GnRH-4Di mice were ovariectomized (OVX) to elevate episodic LH release. Twelve to 20 days later, tail blood for LH assay was sampled at 6-min intervals for 174 min. Mice were sampled for 54 min without treatment, then received an IP saline injection to assess the effects of stress, followed at 114 min by CNO (1 mg/kg, IP, Enzo Life Sciences, Farmingdale, NY or Tocris Minneapolis, MN). LH pulses were detected by a version of Cluster (159) running in IgorPro using cluster sizes of 2 points for both peak and nadir and *t* scores of 2 for detection of increases and decreases.

Experiment 2: CNO can be metabolized to clozapine, which can alter function of central neural systems independent of DREADD receptors (155). To test if clozapine alters LH release, ovary-intact GnRH-Cre mice without either DREADD (n=2) were sampled at 6-min intervals for 114 min, and were given 0.95mg/kg clozapine (Tocris Minneapolis, MN) IP at 54 min. Mean LH before and after clozapine was compared.

Experiment 3: hM3Dq (Dq) To test if activation of GnRH-3Dq targeted to GnRH neurons could increase LH, ovary-intact GnRH-Cre and GnRH-3Dq females (n=3) were sampled at 6-min intervals for 132 min. CNO (1mg/kg) was given IP at 54 min. To test if changes in LH were GnRH-dependent, ovary-intact GnRH-3Dq females (n=2) were injected with the GnRH receptor antagonists Antide (3mg/kg, SC) 45 min before CNO (1mg/kg, IP) administration.

Prepubertal CNO injections and osmospump administration To study the role of manipulating GnRH neuron activity before puberty, GnRH-GFP-4Di or GnRH-GFP-3Dq mice received either intraperitoneal injections of CNO 12 hours apart at a dosage of 0.3mg/kg or a mini-osmotic pump implant (Alzet, 1007D: flow of 0.5 µl per hour for up to 7 days) beginning at two-weeks of age. Osmopumps were filled with 100ul of either 0.5µg/µl CNO in saline with 2.57% DMSO or saline+DMSO vehicle solution. Before surgery, all pups in a litter were removed from the dams' cage including the pups of the CD1 foster dam. Mice were anesthetized with 2-3% isoflurane and a longitudinal incision was made on the back to and the osmospump inserted subcutaneously. Immediately before insertion, the pump was immersed in sterile 0.9% saline solution to ease insertion. The incision was closed with wound clips. Mice were allowed to fully recover before begin placed back with other pups and subsequently the entire litter was returned to dams. Bupivacaine was applied post operatively as a local analgesic and Carprofen (5 mg/kg) was given both just before surgery and 24 h later. At three weeks of age, pups were weaned and either pumps were removed using the same protocol, anesthesia and analgesia, or injections were ceased.

Analysis of reproductive parameters To test if changing postnatal firing of GnRH neurons altered reproductive parameters, day of vaginal opening (VO) was monitored starting after surgery at P14, no mouse had VO before this day. Upon VO, vaginal lavage was used to monitor first estrus. Cycles were assessed by daily vaginal lavage from 9 to 12 weeks of age.

Electrophysiology

Brain slice preparation Solutions were bubbled with 95% O_2/5% CO_2 throughout the duration of experiments and at least 15 minutes prior to tissue exposure to solutions. Slices were made between 8:20-12 EST. The brain was rapidly removed and placed in ice-cold sucrose saline solution containing (in mM): 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 NaH_2PO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal (300 μm) slices were cut with a Leica VT1200S (Leica Biosystems, Buffalo Grove, IL, USA). Slices were incubated in a 1:1 mixture of sucrose saline and artificial cerebrospinal fluid (ACSF) containing (in mM): 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO4, 1.2 $MgSO_4$, 2.5 $CaCl_2$ (pH 7.4) for 30 min at room temperature (21- 23 ºC) and then transferred to 100% ACSF for additional 30 min at room temperature before recording. Recordings were performed 1-5 h after brain slice preparation; no difference in firing patterns were evident based on time after brain slice preparation.

Extracellular recordings Extracellular recordings were made to monitor spontaneous action potential firing (91,92,133) . Slices were placed in a chamber continuously perfused with ACSF (2-3ml/min) and heated to 30-32ºC with an inline-heating unit (Warner Instruments, Hamden, CT, USA). To identify GnRH neurons, an Olympus

(Center Valley, PA, USA) BX51WI microscope was used to briefly illuminate GFP-positive cells in the preoptic area at 488 nm. A Flaming/Brown P-97 puller (Sutter Instruments, Novato, CA USA) was used to pull borosilicate capillary glass (type 7052, 1.65 mm outer diameter; 1.12 mm inner diameter; World Precision Instruments, Inc. Sarasota, FL, USA) into recording micropipettes with a resistance of 2-4 MΩ. Micropipettes used for recordings were filled with HEPES-buffered solution containing (in mM): 150 NaCl, 10 HEPES, 10 glucose, 2.5 CaCl2, 1.3 MgCl2, and 3.5 KCl. All recordings were conducted on one channel of an EPC-10 dual patch clamp amplifier using Patchmaster software (HEKA Elektronik, Pfalz, Germany) running on a Macintosh computer. Low resistance seals (<30MΩ) were formed between the pipette and neuron after first exposing the pipette to the slice tissue in the absence of positive pressure. Recordings were made in voltage-clamp mode with a 0 mV pipette holding potential and signals acquired at 10 kHz and filtered at 5 or 10 kHz. Recording stability and loose seal were checked every 10 minutes by current response to a 5mV hyperpolarizing voltage step. Inactive cells were treated with high-potassium ACSF (20 mM K^+). Cells that exhibited action currents in response were verified to be alive and recordable, and thus those data were used. For cells not responding to K^+, data analysis was truncated at the last observed action current. One-hour recordings of GFP-identified GnRH neurons were made. No more than three extracellular recordings per animal and two cells per slice were included for analysis, and at least four mice from at least three different litters were tested per group. Variation within an animal or among littermates was not less than among all animals within a group. Cell location was mapped to an atlas (135) after recording; no differences in recording parameters were attributable to location.

To test the effect of CNO on GnRH neuron firing, CNO (1μM, 500nM or 200nM) was added to circulating ACSF solution following some of the one-hour extracellular recordings (n=4 for GnRH-GFP-4Di and n= 10 for GnRH-GFP-3Dq).

Whole-cell patch-clamp Pipettes were pulled as described above and resistance was 2-4.5 MΩ when filled with (in mM): 140 KCl, 10 HEPES, 5 EGTA, 0.1 CaCl2, 4 MgATP, and 0.4 NaGTP, 300 mOsm, pH 7.2 with NaOH for recording GABAergic postsynaptic currents (PSCs). Pipettes were wrapped with Parafilm (Bemis) to reduce capacitive transients; remaining transients were electronically cancelled. After achieving a >1 GΩ seal and the whole-cell configuration, membrane potential was held at -60 mV between protocols. Series resistance (Rs), input resistance (Rin), and holding current (Ihold) were measured every 2-3 min using a 5 mV hyperpolarizing step from −60 mV (mean of 16 repeats, 20 ms duration, sampled at 100 kHz and filtered at 10 kHz). Only recordings with a Rin of >500 MΩ, Ihold of −50 to 20 pA, stable Rs of <20 MΩ, and a stable Cm between 8.5 and 23 pF were used for analysis. There were no differences in the passive measurements among groups.

Spontaneous GABAergic postsynaptic currents (sPSCs) were measured in voltage-clamp at a holding potential of -65 mV. Current was sampled at 20 kHz and filtered at 10 kHz. ACSF contained 20 μM D-APV, and 10 μM CNQX to block ionotropic glutamate receptors. At least two 120-second recordings were made for each cell for determining sPSC frequency. To measure activity-independent miniature PSCs (mPSCs), two to three 120-second recordings were made before and during bath application of 1 μM tetrodotoxin (TTX) in a separate set of cells.

Analysis Action currents or PCSs were detected off-line using custom programs in Igor Pro 6.31 and 7.02 (Wavemetrics, Lake Oswego, OR, USA). Extracellular data were binned at 60-sec intervals and were transferred to Excel. Mean firing rate (Hz) and PSC frequency were calculated by dividing the total number of events by the duration of recording. For PSCs, amplitude, interval, rise time, decay, and full-width half-maximum (FWHM) are also reported. Rise time was quantified from baseline to half of the maximum amplitude of the PSC. Decay time was calculated as the time between 90% and 10% of the maximum current amplitude.

Statistics Statistical analyses were performed using Prism 7 (GraphPad Software, La Jolla, CA, USA). Data are reported as individual values with mean±SEM. Data distributions were tested using Shapiro-Wilk. The null hypothesis was rejected if $p<0.05$, but all p values <0.1 are reported. Specific tests were selected based upon experimental design and data distribution and are indicated in the results.

Results

DREADD receptors are effectively targeted to GnRH neurons To verify DREADD expression was present in the expected cells, dual immunofluorescence for GnRH peptide and the HA-tag in the DREADD transgene was performed in GnRH-4Di and GnRH-3Dq mice lacking GFP. HA expression was detected in 94-100% of GnRH-positive neurons (91 of 96 and 93 of 93 for Dq, 112 of 116 for Di). This indicates most GnRH neurons express DREADD. DREADD expression was also noted in non-GnRH neurons, notably in the lateral septum which expresses GnRH during development (160), permitting cre-lox excision of the stop cassette. As a result 42-45% of Dq-positive

and 35% of Di-positive neurons expressed GnRH. Because of the developmental pattern of GnRH expression, this was expected, and indicates off target actions must be considered when interpreting the results.

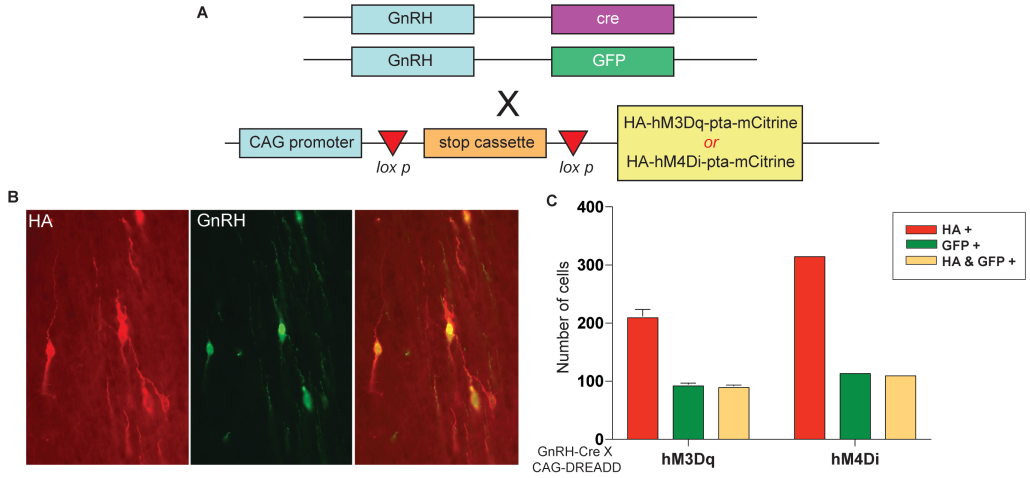

Figure 4-1. Expression of DREADDs in GnRH neurons. A) Breeding scheme for DREADD expression in Cre and GFP expressing GnRH neurons. Mice homozygous for GnRH-Cre and GnRH-GFP were bred to animals expressing either the hM3Dq or hM4Di DREADDS under the CAG promoter. B) Immunofluorescence in a GnRH-cre x Dq-DREADD female adult mouse. Red identifies expression of HA tag on the DREADD, green identifies GnRH. C) Quantification of cells in a brain slice series expressing, HA(DREADD), GnRH and both HA and GnRH (yellow) (n=2 for hM3Dq and 1 for hM4Di).

Activation of DREADDs can alter LH release in GnRH-DREADD mice

Experiment 1: *CNO decreases LH pulses in ovariectomized GnRH-4Di mice.* Baseline LH levels in naturally cycling mice are suppressed by negative feedback, therefore effectiveness of the Di DREADD was assessed in OVX mice, which have elevated LH (Figure 4-2 A-C). In GnRH-Cre mice lacking Di, neither saline nor CNO (1mg/kg) altered LH pulses (n=3, two-way repeated-measures ANOVA/Tukey's). In contrast, in GnRH-Cre-4Di mice, saline had no effect but no LH pulses were observed after CNO injection

(n=3, p<0.0001 vs control and saline within subject, p<0.05 CNO treatment between genotypes, two-way repeated-measures ANOVA/Tukey's). In brain slices, CNO (0.2-1μM) tended to reduce firing rate of GnRH neurons (n=4, 0.9±0.2 Hz before, 0.4±0.2 Hz during, p=0.08 Wilcoxon matched pairs signed rank test).

Experiment 2: *Clozapine does not alter LH.* Other studies have suggested that CNO activation of DREADDs may occur due to metabolism of CNO into clozapine and that some behaviors thought to be CNO-mediated may be effects of clozapine (155). To determine if clozapine alters LH levels independent of DREADD expression, we injected GnRH-Cre mice lacking DREADDS with clozapine (0.95mg/kg, IP). Clozapine had no effect on mean LH (n=2, paired t-test, p=0.5). This indicates changes in LH are likely attributable to CNO activation of DREADD receptors.

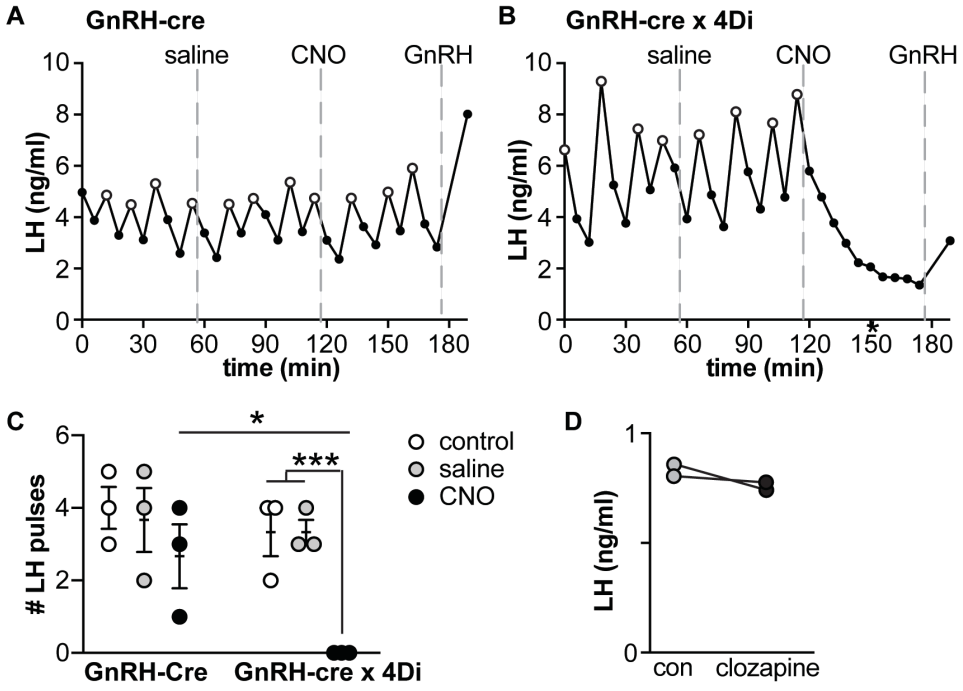

Figure 4-2. Activation of Di-coupled DREADDs targeted to GnRH neurons reduces LH. A, B. LH pulse patterns in OVX GnRH-cre mice (A) or OVX GnRH-cre-4Di mice (B) during a control period, after IP saline injection, after IP CNO (1mg/kg) injection, and

after IP GnRH injection. Cluster detected pulses before GnRH treatment are shown as white symbols, dashed grey lines show times of IP injections. C. Individual values and mean±SEM number of LH pulses during the three treatment periods in A and B, *p<0.05, *** p<0.0001 two-way repeated measures ANOVA/Tukey's (interaction $F(2,8)=5.375$, $p=0.0331$; genotype $F(1,4)=2.42$, $p=0.1948$; treatment $F(2,8)=22.88$, $p=0.0005$; subject $F(4,8)=6.250$, $p=0.0139$. D. Mean of 9 samples before and 9 samples after IP injection of 0.95mg/ml clozapine to control mice (two-tailed, paired t-test, $p=0.5$).

Experiment 3: CNO increases LH release in GnRH-hM3Dq DREADD mice Similar studies were conducted in ovary-intact mice to test if activation of the 3Dq receptor in GnRH neurons increased LH release. In GnRH-Cre mice lacking 3Dq, LH levels remained near baseline after CNO injection (Figure 4-3, n=3, $p=0.97$, two-way repeated-measures ANOVA/Sidak, n=3). In GnRH-cre-3Dq mice, LH levels rose within 6 min of the injection and remained elevated for the remainder of the sampling period (78 min) (n=3, $p<0.01$ two-way ANOVA/Sidak). The CNO elicited increase was GnRH-dependent, as pretreatment of GnRH-cre-3Dq mice with the GnRH receptor antagonist Antide (3mg/kg) blocked the CNO-elicited LH increase (Figure 3-4b, n=2, $p=0.99$, two-way ANOVA/Sidak). Subsequent studies revealed the increase induced by a single CNO injection was maintained for several hours (Figure 4-3c, n=2). Thus CNO effectively activates the reproductive neuroendocrine axis for a prolonged period. In brain slices, CNO increased firing rate of GnRH neurons (n=10, 0.4±0.1Hz before, 1.8±0.4 Hz after, $p<0.01$ Wilcoxon matched pairs signed rank test).

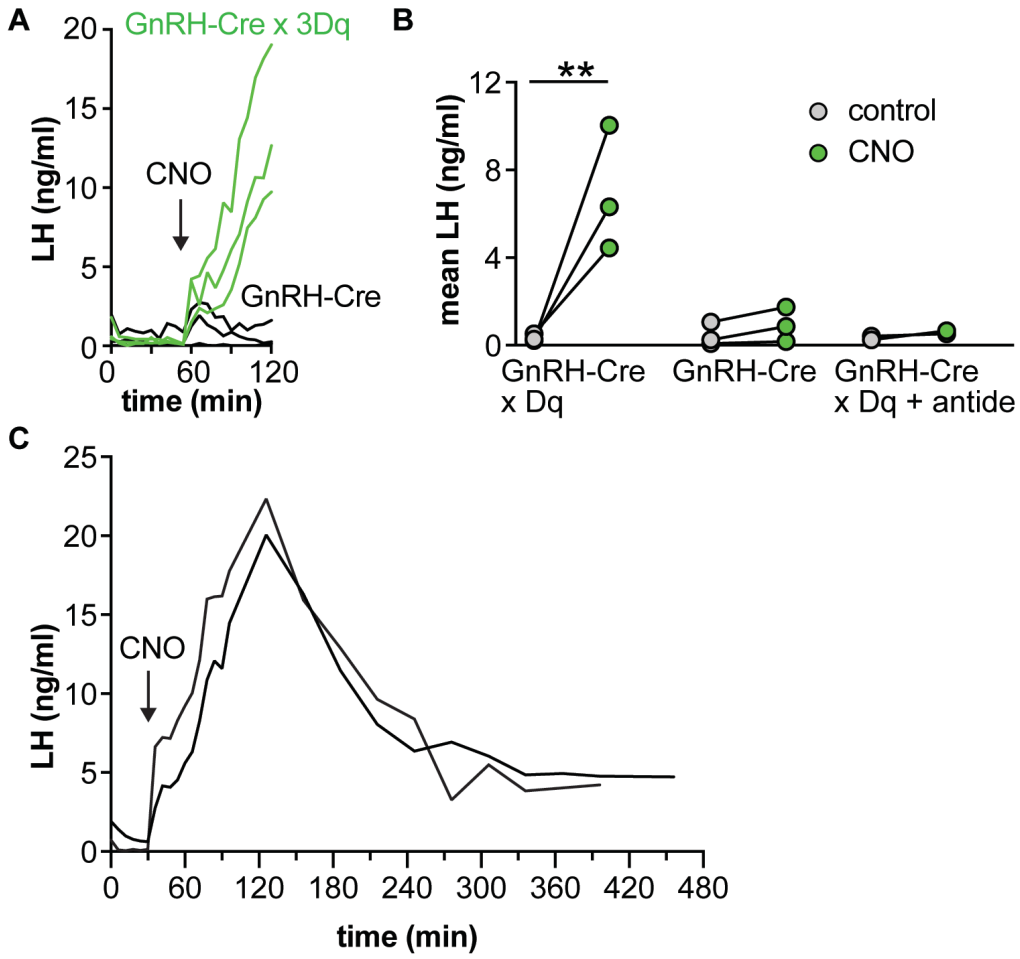

Figure 4-3 CNO induces LH release in GnRH-Cre-Dq mice in a GnRH-dependent manner. A. LH profiles in three GnRH-Cre only controls (black) and three GnRH-Cre-3Dq mice (green) before and after dose CNO injection (1mg/kg) IP (arrow). B. Mean LH in 10 samples before and after CNO injection for left and center, six samples before and after CNO on right. **p<0.01 two-way ANOVA/Sidak. C. Response to CNO injection (0.3mg/kg) remains elevated for several hours after a single injection

Pubertal markers are not affected by altering GnRH neuron activity during prepubertal development. To assess if changing GnRH neuron activity during prepubertal development altered reproduction, three parameters were examined: timing of vaginal opening (the first outward sign of puberty), timing of first estrus, and cycles in adults. CNO was administered via either IP injection every 12h or osmopump from two to three weeks of age. No difference was observed between

treatment methods and these data are combined; osmopumps were judged to be easier on the mice, thus some groups were done entirely with osmopumps. No difference in vaginal opening was observed between CNO and saline treatment in GnRH-GFP-4Di mice (Fig. 4-4A left panel, n=13 each, p=0.5871, two-tailed unpaired Student's t test). Similarly there was no difference in the timing of first estrus between groups (Fig. 4-4B left panel, p=0.1936, n=10 saline, n=9 CNO). In GnRH-GFP-3Dq mice, CNO treatment had no effect in either group (two-way ANOVA, $F(1,63)=0.1843$, $p=0.6692$), but PNA advanced on set of vaginal opening as previously reported (133) (control/saline n=19, control/CNO n=13, PNA/saline n=13, PNA/CNO n=13, $F(1,63)=9.780$, $p=0027$, Fig. 4-4A right panel). Similarly, no differences were found with CNO treatment in the day of first estrus (two-way ANOVA $F(1,49)=0.9750$, $p=0.3283$, Fig. 4-4B right panel). PNA delayed first estrus ($F(1,49)=20.01$, $p= 0.0001$, (control/saline n=19, CNO n=13, PNA/saline n=10, PNA/CNO n=11).

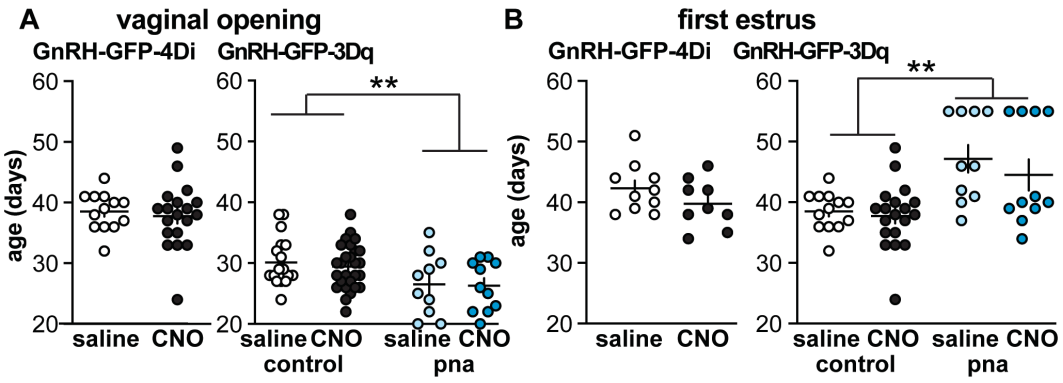

Figure 4-4: *Pubertal markers are not altered by prepubertal CNO treatment but are altered by PNA.* A, B Age at vaginal opening (A) and first estrus (B) in GnRH-GFP-4Di (left) and vehicle and GnRH-GFP-3Dq (right) mice. *** p<0.01 PNA vs control, two-way-ANOVA.

Reproductive cycles are not affected by alterations in prepubertal GnRH neuron firing: Estrous cycles were assessed from 9-12 weeks of age to determine if they were altered by modulation of prepubertal GnRH neuron activity. Representative cycles from each group are displayed in Figure 4-5 A, B and quantification of days spent in each cycle stage are in Figure 4-5 C. In GnRH-GFP-4Di mice, there was no difference between CNO and saline treatment at any cycle stage (two-way ANOVA, $F(1,45)=0.000$, $p>0.9999$). In GnRH-GFP-3Dq mice, PNA mice spent more days in diestrus and estrus than control mice (two-way ANOVA/Tukey's, $p<0.001$ all groups). No difference between CNO and saline treatment at any cycle stage were detected in GnRH-3Dq mice (two-way ANOVA/Tukey's $p>0.14$). CNO-treated PNA mice had slightly fewer days in proestrus than CNO-treated control mice ($p<0.05$, two-way ANOVA, Tukey's). Thus PNA altered reproductive cycles but CNO mediated activation had minimal effect.

Prepubertal alteration of GnRH firing rate alters neurobiology in adults. To assess if changing GnRH neuron firing during the prepubertal period alters adult GnRH neuron firing, hour-long extracellular recordings were conducted. Treatment with the vehicle had no effect on firing in either genotype (Figure 4-6). In GnRH-GFP-4Di mice (Figure 4-6A), CNO treatment from two to three weeks of age increased GnRH neuron firing in adults (two-tailed Mann-Whitney U-test, $p<0.01$). This result is similar to what is observed in PNA mice where decreased prepubertal GnRH neuron activity is coupled with elevated GnRH neuron activity in adulthood. This treatment has a strong preliminary trend to reduce frequency of GABAergic transmission to GnRH neurons in adults (Figure 4-6B,); this is opposite to what was previously observed in adult PNA mice(58). In GnRH-GFP-3Dq mice (Figure 4-7), preliminary results suggest CNO

treatment from two to three weeks of age may reduce GnRH neuron firing in PNA but not control adults.

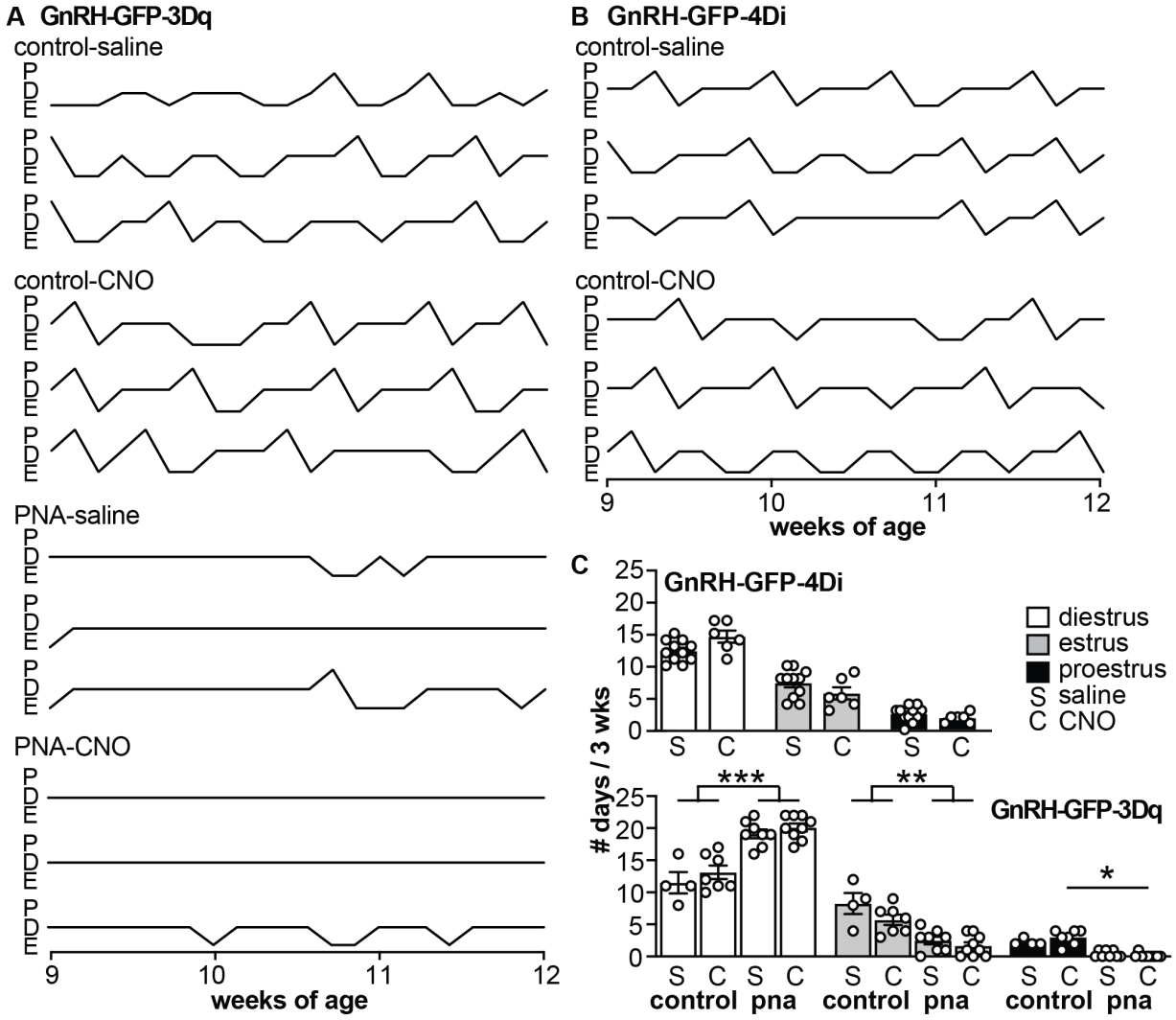

Figure 4-5. PNA treatment but not altering GnRH neuron activity during development alters estrous cycles. A, B, representative cycles from the different groups. C. Individual values and mean±SE of number of days spent in each cycle stage. Top GnRH-GFP-4Di mice, bottom GnRH-GFP-3Dq mice. ***p<0.0001 control vs PNA; *p<0.05 CNO control vs CNO PNA GnRH-GFP-3Dq.

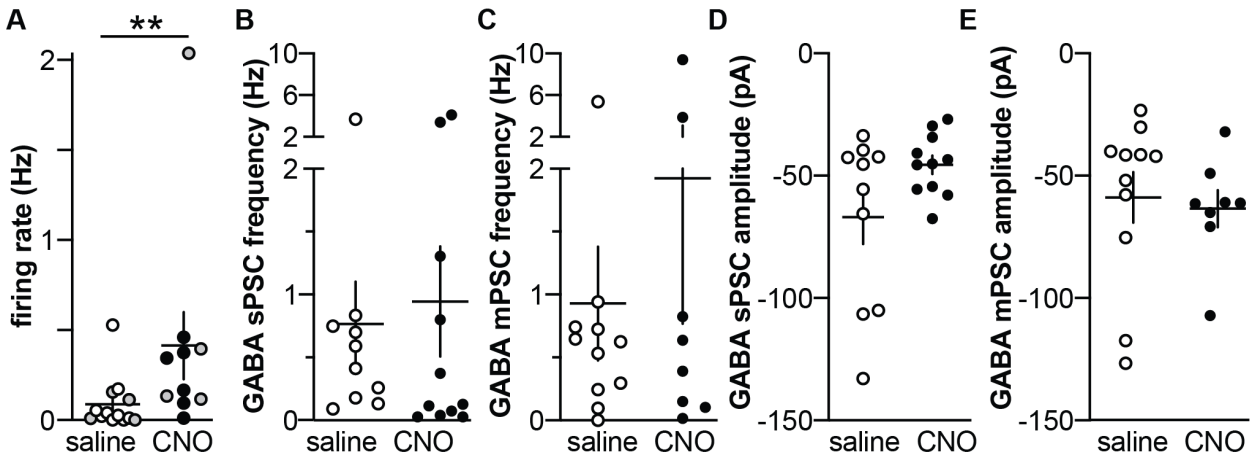

Figure 4-6. Reducing GnRH neuron activity from two to three weeks of age changes GnRH neuron firing rate but not GABAergic transmission in adults. A-E Individual values and mean±SEM GnRH neuron firing rate (A),GABA sPSC frequency (B) GABA mPSC frequency (C), GABA sPSC amplitude (D) GABA mPSC amplitude (E) in GnRH-GFP-4Di mice. *p<0.01 two-tailed Mann-Whitney U test. Grey symbols in A indicate mice receiving twice daily IP injections of CNO or saline, open symbols osmopump administration of saline and black symbols osmopump administration of CNO.

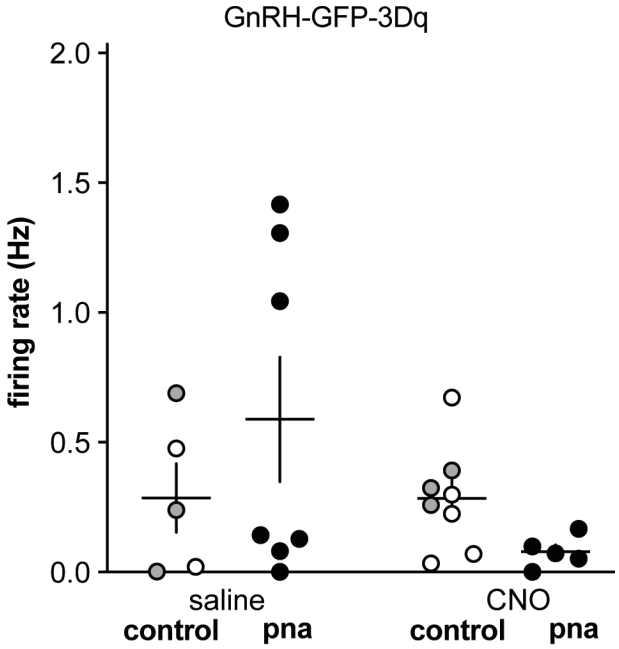

Figure 4-7. Preliminary data indicate increasing GnRH neuron activity from two to three weeks of age may reduce adult GnRH neuron firing rate in PNA adults. Grey symbols indicate mice receiving twice daily IP injections of CNO or saline, open symbols osmopump administration of saline and black symbols osmopump administration of CNO

Discussion

Our recent work demonstrated central components of the reproductive axis are active prior to the outward signs of puberty. Further, in prenatally androgenized (PNA) mice, prepubertal neurobiological changes before puberty are correlated with later adult reproductive dysfunction. Those studies, however, could not separate the effects of PNA treatment from subsequent changes in GnRH neuron activity. Here we utilized chemogenetics to study the role of prepubertal activity in GnRH neurons on later reproductive function. We found that while altering prepubertal GnRH neuron activity is sufficient to change the adult GnRH neuron firing rate, it is not sufficient to affect reproductive parameters.

To our knowledge, this is the first study using DREADD expression in GnRH neurons to modulate their activity. Changes in LH release *in vivo* were as hypothesized for the respective DREADD utilized, i.e., increased LH with 3Dq activation in GnRH neurons and reduced LH with 4Di activation. The GnRH-cre effectively targeted expression of both DREADDs to GnRH neurons, with >95% of these cells expressing these receptors. There was expression of DREADDs, however, in non-GnRH neurons, particularly the lateral septum, thus we cannot exclude possible off-target effects from CNO action via these neurons in the changes observed. Importantly, CNO was able to alter GnRH neuron firing rate in the expected direction in brain slices made from adult mice as well as mice aged two to three weeks.

The present work indicates that neither chemogenetic activation nor suppression of GnRH neurons from two to three weeks of age alters timing of vaginal opening or first estrus. Possible explanations for this are that the activity manipulation is not sufficient to

cause a permanent change in the network, and/or that this manipulation is at the wrong time during development to result in later physiological changes. The importance of timing of neuronal activity in establishing adult function was demonstrated in the visual system, in which there is a critical period of postnatal activity required for proper synaptic innervation of the primary visual cortex (161). In this regard, the present treatment period was chosen based on when PNA-induced alterations in GnRH neuron firing rate occur, and is at an age when activity-dependent synaptic changes have been shown to be important in other systems (162,163). It is also possible that the chemogenetic treatment was too proximal to vaginal opening to alter its timing. PNA treatment reduces GnRH neuron activity at this same time period, and advances vaginal opening, but these activity changes are subsequent to androgen treatment (133). Together these observations suggest that changing GnRH neuron activity alone is not sufficient to alter pubertal markers and that prenatal programming actions of androgens are needed.

While altering prepubertal GnRH neuron activity was not sufficient to change reproductive parameters, changing this activity did alter adult GnRH neuron function. Specifically, inhibition of GnRH neuron activity during development increased firing rate, of these cells in adults. This supports what was observed in the PNA model in which decreased GnRH neuron firing rate prior to puberty is correlated with increased firing rate in adulthood, and extends that observation to demonstrate causation between early and later changes in firing rate. In PNA mice, suppressed firing rate before puberty is also correlated with increased GABAergic input in adulthood. No change, however, was observed in spontaneous or miniature GABAergic input to GnRH neurons in mice in

which prepubertal GnRH neuron activity was suppressed. This suggests increased GABAergic input in PNA mice is at least in part attributable to androgen action. Of interest in this regard, DHT can increase spine density of CA1 hippocampal neurons in adult ovariectomized female rats (164), and prepubertal exposure to androgens in male rats also increased spine density in these cells (165). Taken together with evidence from PNA mice suggesting synaptic sites on GnRH neurons are increased in these animals, this raises the possibility that androgens may play a stronger role in determining inputs than GnRH neuron activity. Further, while GABA is the primary fast synaptic input to GnRH neurons, these observations do not rule out changes in intrinsic excitability, glutamatergic inputs or those of peptidergic neurons.

Data on the effects of activating GnRH neurons from two to three weeks of age are still being collected and must be interpreted with caution due to the current low number of recordings. Preliminary data suggest however that DREADD activated GnRH neurons in PNA mice, but not controls, may exhibit reduced firing rate as adults. This would suggest that increasing activity that is prepubertally lower in PNA animals could ameliorate the changes observed in GnRH neuron firing rate in PNA mice.

Reproductive cyclicity is disrupted in PNA mice, which also exhibit altered prepubertal activity of GnRH neurons. We therefore hypothesized that changing activity during development would alter firing rate in adulthood and thus modulate reproductive cycles. While changing firing rate in young mice appears to alter adult GnRH neuron firing rate, no changes in cyclicity were induced in these mice. These results suggest that there are compensatory mechanisms that overcome changes in GnRH neuron activity during development, that there is a wide range of GnRH neuron firing rates that can support

reproduction, and/or that the aspect of the PNA treatment that is critical for disrupting cycles is not reproduced by solely changing GnRH neuron activity during development. It is important to note that GnRH neurons are thought to be unable to respond directly to sex steroids due to lacking most steroid receptors (124,166). In this regard, arcuate kisspeptin neurons, which are directly androgen responsive, may play a critical role in generating the input to GnRH neurons needed to regulate cyclicity. Deletion of estrogen receptor alpha in arcuate kisspeptin neurons disrupts cyclicity (167,168). Future studies should thus address the functional role such upstream cell population may play in reproductive deficits found in PNA mice.

Our results suggest that, in the absence of prenatal androgen exposure, a range of prepubertal GnRH neuron activity may be acceptable for setting up the network in a manner that permits typical estrous cycles. They also suggest that androgens play a necessary role in disrupting reproductive parameters in both the PNA model and, perhaps, in PCOS. Supporting the latter are similar observations in many models that utilize different methods to mimic increased androgens, including prenatal treatments with testosterone or anti-Mullerian hormone, and postnatal treatment with letrozole, which inhibits aromatase, the enzyme that converts androgens to estrogens. Also supporting the strong role of androgens in controlling GnRH neuron activity in PNA mice is the results of Chapter 3. Together these results show that androgens play a role at least in adult firing rate of GnRH neurons in both control and PNA mice.

Chapter 5: The role of androgens and prepubertal GnRH neuron activity in determining adult reproductive function in control and PNA mice

This dissertation project focused on the role of prepubertal activity of GnRH neurons and its modification by a model commonly used to study mechanisms underlying a prevalent reproductive disorder in humans. We observed that GnRH neurons are indeed quite active during the prepubertal period and that prepubertal activity is modified in PNA mice. We further examined the roles of androgens in producing and maintaining these differences in prepubertal and adult animals.

Prepubertal activity of GnRH neurons

In Chapter 2, GnRH neuron activity across prepubertal development was characterized. The finding that activity was sexually differentiated informs our understanding of reproductive development and pubertal timing between sexes because it demonstrates differences in the GnRH neuron network between males and females even before puberty. Differences in prepubertal GnRH neuron activity between males and females also support a role for early androgen exposure in development of sexually differentiated neuronal circuits governing reproductive function. This idea is further supported by studies in which perinatal exposure to testosterone propionate in female rats caused the volume of the sexually dimorphic nucleus of the preoptic area to increase to male levels (169). While our findings support that prepubertal GnRH neuron firing rate in female PNA mice was more similar to males than control females, finer

aspects of firing, such as bursts, showed that activity patterns were not fully masculinized. Another argument against full masculinization of the GnRH neuron network occurring in PNA females is the observation that LH surges can still be induced by estradiol in PNA mice (60). Further, GABAergic input to GnRH neurons is different between males and PNA females (134). Taken together these data suggest that while certain aspects of the GnRH neuron network in PNA mice may be more “male-like”, PNA induces a unique organizational profile.

GnRH neurons are the final central mediators of reproductive function, but receive considerable input from the afferent network. One caveat of the present studies is that prepubertal changes in upstream cells that may or may not be directly androgen responsive were not examined. GABAergic neurons are major contributors to GnRH neuron function in regard to steroid feedback necessary for reproduction (28,170,171). Both firing rate of and GABAergic input to GnRH neurons peaked at three weeks of age in control females (134), suggesting three weeks of age as an important time in the course of establishing central control of reproductive function in females. Furthermore, observations have also shown that functional GABAergic input to GnRH neurons is increased in PNA mice at three weeks of age (134) and that PNA mice display increased GABAergic appositions to GnRH neurons by postnatal day 25 (61). In adults, GABA-expressing cells in the arcuate nucleus more strongly project to GnRH neurons in PNA mice (59). While the neuropeptide identity of those arcuate cells is still being defined, this population of neurons is of interest in future studies investigating functional disruptions of brain circuits in PNA animals.

Overall, Chapter 2 demonstrates that GnRH neurons are active during prepubertal development and that PNA disrupts this activity. This altered activity may underlie changes in the reproductive axis even prior to when outwards signs of puberty and full adult reproductive function are present.

Ovarian factors and their regulation of GnRH neuron firing in PNA mice

Ovarian feedback mechanisms are commonly altered in both women with PCOS and animal models that recapitulate aspects of this syndrome. Previous studies in our lab investigated how steroid milieu affects both firing rate of and GABAergic inputs to GnRH neurons in control adults (172). However, ovarian effects on GnRH neuron firing had not been studied in the PNA mouse model. To help understand the differences between GnRH neuron firing rate in three week old and adult PNA animals, and to further link ovarian function to our observations of GnRH neuron firing rate, in Chapter 3 we studied the role of the ovary and androgens in GnRH neuron firing of PNA mice.

In adults, ovariectomy removes negative feedback in controls, leading to increased GnRH neuron firing rate. Our studies in PNA mice, however, demonstrate GnRH neuron firing rate was drastically decreased upon ovarian removal. This result was unexpected when considered in the context of work that suggests that steroid feedback at a central level is disrupted in PNA models and PCOS women. In hyperandrogenemic women with PCOS, progesterone negative feedback is impaired and thus an upregulation of hypothalamic output, as reflected by an increase in LH pulses, is observed. In other PNA animal models, such as in sheep and monkeys, disruption of ovarian feedback has also been observed (51,52). Also, PNA mice display lower levels of progesterone

receptor expression in the hypothalamus compared to controls (59). Taken in light of these studies, one might expect firing rate upon removal of the ovary in PNA mice to stay elevated or perhaps further increase. In our studies however firing rate decreased suggesting that ovariectomy in PNA mice may remove an activating factor rather than feedback that negatively regulates hypothalamic and pituitary output.

It has been previously demonstrated that androgens have an activating effect on the reproductive axis. In PCOS, upregulation of neuroendocrine output is thought to be driven by the presence of elevated, yet sub-male, levels of androgens (173). In castrated mice, DHT given in combination with estradiol has been demonstrated to increase GnRH neuron fringing rate when compared to estradiol alone (29,57). These studies suggest that ovarian androgens may play a role in increased GnRH neuron firing rate in PNA mice. Our findings that DHT increased firing in both control-OVX and PNA-OVX mice also support this postulate. One caveat of our studies is that androgens are not the only ovarian factor that contribute to GnRH feedback. These studies do not investigate other ovarian factors that may be acting to modulate upstream reproductive networks in PCOS and PNA animals such as estradiol and progesterone.

In PNA-OVX mice, circulating LH levels did not correspond to a decrease in GnRH neuron activity. A caveat of these measurements is that they are single point LH measurements. Because LH is released in pulses, these values could represent LH levels at the peak or trough in a pulse rather than a representative value at baseline. Taken together with the observation of low GnRH neuron firing rate in PNA-OVX mice, LH values reflect that pituitary but not central feedback may still be intact in these mice. This is similar to examples in sheep exposed to prenatal androgens. In one study, it was

found that PNA sheep commonly do not display LH surges in response to estradiol-induced positive feedback; however this study also demonstrates that a lack of LH surges was not due to pituitary insensitivity to GnRH (174). Also in PNA mice, intact positive feedback at the level of the pituitary has been suggested by the observation PNA mice are able to mount an estradiol-induced LH surge (60). These studies suggest that some pituitary feedback mechanisms may still be intact in PNA animals even if local changes in the GnRH neuron network are observed.

Overall, these studies suggest that ovarian androgen action is, at least in part, responsible for an upregulation in output of the GnRH neuron network in PNA adults. The emergence of an effect of ovariectomy on GnRH neuron firing rate in PNA adults suggests a role for central prenatal programming in driving later changes in GnRH neuron physiology. Thus, follow-up studies should consider investigation of the effects of prenatal androgen exposure on both intrinsic properties of the GnRH neuron and on upstream neuronal populations where ovarian androgens may be directly acting to drive abnormal reproductive function in PNA mice.

Prepubertal alteration of GnRH neuron activity and reproductive outcomes

In Chapter 4 we sought to investigate if changing prepubertal GnRH neuron activity alone could alter later reproductive function and the adult GnRH neuronal network. Interestingly, changing activity during the prepubertal period was sufficient to change adult GnRH neuron activity but not reproductive parameters. This finding suggests that more than prepubertal GnRH neuron activity changes are required to alter reproductive outcomes in both control and PNA mice.

While no alterations in reproductive parameters were noted, these studies do not fully rule out a role for prepubertal GnRH neuron activity in establishing later reproductive function. There are indeed several caveats of these studies. Firstly, these studies only altered prepubertal GnRH neuron activity from the end of the 2nd to the start of the 3rd postnatal week. Previous studies on which this time period was based indicate that changes in GnRH neuron activity occur in control vs PNA females at this time (133), however they do not give insight to what may happen between these points in development. There may in fact be an unobserved critical period where GnRH neuron activity leads to the correct setup of the GnRH network. In other systems, manipulations during the critical periods result in later observations of changed neuronal output of the system of interest (175). For example, studies in the mouse visual system demonstrate that ocular dominance is most strongly shifted after monocular deprivation when it is induced between postnatal days 28 and 32 (176). While a critical period for GnRH neurons has yet to be defined, changes in synaptic spine density of GnRH neurons in rodents have been noted across pubertal maturation (between P10 and P90) (177,178). Taken together with the firing rate data from the aforementioned study(133), this suggests our manipulation of GnRH neuron firing rate occurred a time when GnRH neuron plasticity is indeed occurring.

A second caveat to these studies is that the DREADD approach does not fully recapitulate activity fluctuations that may play a role in network changes in PNA mice. GnRH neurons show different variations of activity patterns including bursts (94) that may be important for development as shown in other systems. For example, in the *Drosophila* visual system cells possess a unique activity signature that is thought to

coordinate development of the adult neuronal circuity (179). Thus fluctuations of activity in GnRH neurons may also play a role in network changes that lead to reproductive dysfunction found in PNA mice. Finally, despite changes in GnRH neuron firing induced by chemogenetic activation during the postnatal period, compensatory mechanisms may overcome these manipulations once CNO is removed. Studies investigating memory formation in male mice, support that alteration of neuron dendritic spine density and connectivity can occur in hippocampal neurons in adulthood (11-15 weeks) (180). Evidence for structural changes in adult GnRH neurons have been observed. For example, in mice where an estradiol induced LH surge had been elicited, c-fos positive GnRH neurons showed a robust increase in spine density (181). Therefore altered structural connections in the GnRH neuron circuitry brought about by manipulating early activity could potentially undergo later remolding that allows successful reproductive function to occur.

As a final point, both firing rate and androgen levels are altered in PNA mice. This makes it difficult to ascertain which of these variables contributes to the noted reproductive phenotypes. Although studies in Chapter 4 did not focus on androgens or manipulate them in any way, a lack of changes in reproductive parameters supports that androgens may have a stronger influence than GnRH neuron firing on disrupted reproductive function in PNA mice.

Summary

This dissertation project advances our understanding of the prepubertal GnRH neuron network in both control and prenatally androgenized mice. It identifies that GnRH

neuron activity occurs prepubertally in both males and females before outward signs of reproduction are present. It demonstrates that this early prepubertal activity is disrupted in prenatally androgenized mice and also reveals that androgens may program early organizational alterations that lead to later activational effects on GnRH neuron firing in adult PNA mice. Most excitingly, it utilizes a chemogenetic approach to alter prepubertal activity in GnRH neurons and finds that this alteration can lead to a later change in GnRH neuron firing.

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