Development of GABA Inputs to Gonadotropin-Releasing Hormone (GnRH) Neurons and Effects of Prenatal Androgen Exposure

by

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Dedication

For my family, who taught me how to climb mountains and to ski down the other side and have always been my rock.

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Sue, who has taught me so much and then taught me again, ‘Festina lente’.

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Abstract

Polycystic ovary syndrome (PCOS) is a leading cause of infertility and is characterized by hyperandrogenemia, polycystic ovaries and irregular menses. The underlying cause of PCOS is unknown but may involve effects of androgens during prenatal development. Women with hyperandrogenemia during pregnancy have daughters at higher risk of developing PCOS as adults. Androgens may interfere with development of neuronal networks important for regulating reproduction. Gonadotropin-releasing hormone (GnRH) neurons are the final common pathway for the central regulation of fertility. Pulsatile GnRH release signals the pituitary to release luteinizing hormone (LH) which stimulates ovarian hormone production. In most women with PCOS, GnRH/LH pulse frequency is persistently elevated, contributing to excess ovarian androgen production and irregular cycles. Clinical studies have revealed that obese peripubertal girls with hyperandrogenemia have elevated LH pulse frequency suggesting that neuroendocrine dysfunction seen in adult women with PCOS emerges during prepubertal development.

Prenatal androgen (PNA) exposure has been used in experimental models to replicate aspects of PCOS in adulthood. GnRH neurons from adult PNA mice have increased firing rate and increased excitatory GABAergic transmission. GABAergic afferents are a major source of input to GnRH neurons but neither the typical pattern of when GABAergic transmission develops nor when changes emerge with PNA are understood. We used whole-cell voltage clamp recordings to examine GABAergic postsynaptic currents (PSCs) in GnRH neurons from control and PNA

mice during postnatal development in both females and males. 1-wk old female and male mice had low frequencies of GABAergic transmission that increased to adult levels by 3- and 4-wks of age, respectively. This increase was more robust in PNA mice of both sexes. This difference persisted into adulthood in females but not males. Differences in PSC frequency were activity independent, consistent with changes in synaptic connectivity rather than presynaptic activation. In PNA females, increased GABAergic transmission occurs concomitantly with decreased GnRH neuron firing at 3-wks of age. The percentage of cells firing and membrane potential depolarization in response to GABA was decreased in PNA females at 3-wks of age but not adults. This difference was not due to altered chloride homeostasis of GnRH neurons as the equilibrium potential of the $GABA_AR$-mediated current was not altered by PNA at 3-wks of age. Our work shows that prenatal androgen exposure programs neuroendocrine changes that may contribute to adult reproductive dysfunction. Increasing numbers of pre-adolescents are at risk for developing hyperandrogenemia in association with childhood obesity, highlighting our need for greater understanding of how excess androgen exposure affects developing brain circuits.

Chapter 1 Introduction

Polycystic ovary syndrome [PCOS] is a leading cause of infertility in women around the world (Goodarzi et al., 2011). In addition to infertility, PCOS has profound life-long health implications including an association with metabolic syndrome and increased risk of hospitalization for heart disease, diabetes, stroke, cancer and mental health disorders (Hart and Doherty, 2015). In the US, approximately 8% of women of reproductive age are affected by PCOS as defined by the classic 1990 NIH diagnostic criteria of irregular or absent menstrual cycles and hyperandrogenemia (Zawadski and Dunaif, 1992; Bulletins--Gynecology, 2009). These diagnostic criteria were expanded in 2003 to include women who met any two of the following three features: 1) irregular or absent menstrual cycles, 2) hyperandrogenemia, or 3) polycystic ovaries (Rotterdam, 2004). This introduction will primarily focus on the classic NIH-defined PCOS phenotype as it most clearly relates to neuroendocrine features of PCOS that will be examined in the following work. The absence of unified diagnostic criteria reflects the broad range of clinical presentations of PCOS and has impeded discovery into the basic mechanisms and therapeutic interventions for the disease.

Despite the high prevalence and impact of PCOS, the etiology and mechanism(s) of disease progression are still incompletely understood. It has long been recognized that PCOS tends to run in families, suggesting that a degree of genetic susceptibility and/or environmental factors may contribute to PCOS pathology (Kahsar-Miller et al., 2001). Genome wide association

studies have identified several loci of genetic susceptibility however the predictive power of these variants is low, indicating that genetic heritability alone does not account for the familial clustering of PCOS (Brower et al., 2015). Androgen exposure during development has been proposed as an environmental risk factor for PCOS. Women with congenital adrenal hyperplasia (CAH) have very high levels of androgens and their daughters are at high risk for developing PCOS as adults (Hague et al., 1990). Women with PCOS also have elevated androgens during pregnancy and their daughters have elevated testosterone levels during later stages of pubertal development and are at increased risk of developing PCOS themselves (Sir-Petermann et al., 2002; Kent et al., 2008; Maliqueo et al., 2009; Sir-Petermann et al., 2009). Obesity during prepubertal development is associated with elevated androgen levels, which may promote the onset of PCOS in these girls (McCartney et al., 2006; Knudsen et al., 2010).

The central neuroendocrine system that helps regulate reproductive function is persistently overactive in most adult women with PCOS (Rebar et al., 1976). Clinical studies have revealed the presence of neuroendocrine features of adult PCOS in hyperandrogenemic prepubertal girls (Chhabra et al., 2005; Blank et al., 2009). The outward clinical markers of PCOS typically appear during puberty, suggesting that underlying mechanisms of PCOS arise prior to pubertal maturation (Franks, 2008; Knudsen et al., 2010). Neuroendocrine changes during prepubertal development likely have important implications for adult reproductive function and may play a role in the etiology of PCOS. This introduction will review aspects of the typical neuroendocrine regulation of reproductive function, how changes within this system may contribute to PCOS pathology and the role of changes during pre- and peripubertal development in the progression of

PCOS. This introduction will be limited to studies published before the start of my PhD work in 2014.

Neuroendocrine regulation of reproductive function (HPG axis)

In vertebrates, reproductive function is regulated through interactions of the hypothalamus, pituitary and gonads. Gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus are the final common pathway for the central regulation of reproduction. GnRH neurons extend processes to the median eminence and release GnRH close to the hypophyseal portal capillaries leading to the anterior pituitary. GnRH is released in discrete pulses (Carmel et al., 1976; Moenter et al., 1992). Gonadotropes in the anterior pituitary are sensitive to the frequency of GnRH release; high and low frequency GnRH pulses signal gonadotropes to preferentially synthesize and release the gonadotropin hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH), respectively (Wildt et al., 1981; Haisenleder et al., 1991). LH and FSH promote gonadal steroidogenesis and ovarian follicle maturation (Bousfield, 2006). Pulsatile GnRH is both necessary and sufficient for fertility; pituitary gonadotropes become refractory to continuous non-pulsatile GnRH (Belchetz et al., 1978) and administration of pulsatile GnRH can restore menstrual cycles in individuals with hypogonadotropic hypogonadism (Martin et al., 1990). Pulses of GnRH in portal blood are closely associated with pulses of LH in the peripheral circulation, allowing LH to be used as a convenient bioassay for the pattern of GnRH release (Clarke and Cummins, 1982).

Shifts in the pattern of GnRH release regulate reproductive cycles. GnRH pulse frequency and the gonadotropin response to GnRH are in turn modulated by ovarian steroid hormone feedback

to the hypothalamus and pituitary (Clarke and Cummins, 1984; Karsch, 1987; Shaw et al., 2010). During most of the length of the reproductive cycle, estradiol has a negative feedback effect on GnRH/LH pulses (Reame et al., 1984; Chongthammakun and Terasawa, 1993). Estradiol levels peak during the late follicular phase (proestrus in rodents). During this period, the central effect of estradiol feedback undergoes a switch from negative to positive (Filicori et al., 1986; Evans et al., 1994; Czieselsky et al., 2016). These changes lead to increased GnRH neuron firing rate and a surge of GnRH release (Sarkar et al., 1976; Moenter et al., 1990; Christian et al., 2005; Glanowska et al., 2012). The GnRH surge induces an LH surge which triggers ovulation (Docke and Dorner, 1965). Following ovulation, progesterone is secreted by the resulting corpus luteum and is associated with a marked decrease in LH pulse frequency and amplitude (Filicori et al., 1986; Rossmanith et al., 1990).

Progesterone feedback is an important modulator of GnRH/LH pulse frequency. Progesterone treatment of naturally cycling women resulted in lowered LH pulse frequency and decreased mean plasma LH levels compared to untreated women at all phases of the menstrual cycle (Soules et al., 1984). In another study of naturally cycling women, treatment with estradiol in addition to progesterone was necessary to prevent the increase in LH pulse frequency from the luteal to follicular phase (Nippoldt et al., 1989). Estradiol regulates progesterone receptor expression in the hypothalamus and thus likely helps enable progesterone negative feedback (Romano et al., 1989). The relative contributions of estradiol and progesterone feedback effects on LH pulses were assessed by treating ovariectomized rats with implants of either estradiol, progesterone or both (Leipheimer et al., 1984). Ovariectomy (OVX) resulted in a sharp increase in pulsatile LH that was suppressed by progesterone alone but was unaltered by estradiol

treatment alone. Progesterone treatment in combination with estradiol had an even more robust suppression of LH pulse frequency and amplitude. Interestingly, this effect may be mediated, at least in part, by the hypothalamus as the pituitary response to GnRH was not different between OVX rats with or without progesterone. Similar effects of combination progesterone and estradiol treatment on LH pulses have been observed in OVX sheep, further highlighting the synergistic role of estradiol to promote central sensitivity to progesterone negative feedback (Goodman et al., 1981).

Neuroendocrine dysfunction in polycystic ovary syndrome

In most women with PCOS, LH pulse frequency is persistently elevated compared to typically cycling women (Rebar et al., 1976). This likely reflects elevated GnRH pulse frequency as the pulse frequency of LH mirrors the pattern of episodic GnRH release (Moenter et al., 1992). Persistently elevated GnRH pulse frequency leads to an increased LH to FSH ratio that is characteristic of PCOS. This altered gonadotropin signaling, together with changes in ovarian physiology, contribute to elevated androgen levels in PCOS women (Goodarzi et al., 2011; McCartney and Marshall, 2016). First, thecal cells from polycystic ovaries are primed to secrete excess androgens in response to LH (Gilling-Smith et al., 1994). Second, the altered gonadotropin ratio results in immature follicles that are deficient in aromatase, further exacerbating hyperandrogenemia (Taylor et al., 1997). Treatment of women with PCOS with a long-acting GnRH agonist lowered their serum LH and this appears to normalize their serum androgen levels, suggesting that high LH pulse frequency drives ovarian hyperandrogenemia in these women (Chang et al., 1983). Furthermore, GnRH antagonists can reduce testosterone levels in women with PCOS, indicating that abnormal gonadotropin secretion in PCOS is likely to be at

least in part due to hypothalamic dysfunction (Hayes et al., 1998). Regardless as to whether elevated GnRH pulse frequency is a primary cause or a consequence of PCOS, it perpetuates the hyperandrogenemia that is characteristic of the disease.

Elevated LH levels in women with PCOS may be secondary to loss of ovarian feedback during anovulatory cycles and/or to central dysfunction. To examine if elevated LH levels in PCOS are a consequence of lack of progesterone exposure, oral contraceptives (OC) were given to women with PCOS and to eumenorrheic women to stimulate luteal phase estrogen and progesterone levels. During OC treatment LH pulse frequency was elevated in women with PCOS compared to eumenorrheic women and this difference persisted after OC treatment was withdrawn, suggesting that central dysfunction in addition to lack of progesterone exposure drives the elevated LH:FSH ratios in PCOS (Daniels and Berga, 1997). Interestingly, women with PCOS treated with a 21-day course of estradiol and progesterone at levels typically seen during the luteal phase showed a delayed and less robust suppression of LH pulse frequency and amplitude compared to control females (Pastor et al., 1998). In a follow-up dose-response study, women with PCOS also required higher plasma progesterone levels to suppress LH frequency to the same degree as seen in control women with similar plasma progesterone levels (Pastor et al., 1998). These data suggest that the hypothalamic response to ovarian feedback, not just altered hormonal milieu itself, contributes to abnormal gonadotropin secretion in PCOS.

Effect of hyperandrogenemia on progesterone feedback

Impaired sensitivity to progesterone feedback in PCOS may be an effect of elevated androgens. Adult women with elevated androgens due to congenital adrenal hyperplasia (CAH) have increased LH pulse amplitude compared to controls (Levin et al., 1991). Female primates

exposed to mildly elevated levels of testosterone during pubertal development showed increased LH pulse frequency and LH responsiveness to GnRH (McGee et al., 2012). These findings suggest that hyperandrogenemia can disrupt gonadotropin secretion. Adult female mice treated with testosterone showed blunted estradiol-induced progesterone receptor expression, suggesting impaired progesterone feedback as one potential mechanism through which elevated androgens could exacerbate the altered gonadotropin levels in PCOS (Foecking and Levine, 2005). Recordings of GnRH neuron firing in mouse brain slices have examined the effect of androgens on progesterone and estrogen feedback. In female OVX mice, treatment with combined progesterone and estradiol decreased GnRH neuron firing rates compared to treatment with estradiol alone (Pielecka et al., 2006). In contrast, treatment with combined androgen and estradiol increased GnRH neuron firing rate and the frequency of peaks in the firing rate compared to treatment with estradiol alone. This increase in GnRH firing rate persisted even with the addition of progesterone, suggesting that androgens interfere with progesterone negative feedback to GnRH neurons (Pielecka et al., 2006). Of interest in this regard, treatment with the androgen receptor antagonist flutamide for four weeks did not itself suppress either LH pulse frequency or LH responsiveness to GnRH in women with PCOS. However, flutamide treatment did restore sensitivity to progesterone negative feedback (Eagleson et al., 2000). Androgens may act through multiple mechanisms to alter GnRH neuron function, including through the upstream GnRH neuronal network.

GABAergic network afferent to GnRH neurons

Ovarian hormone feedback is likely conveyed to GnRH neurons through their afferent network. GnRH neurons themselves do not appear to express detectable levels of most ovarian steroid

receptors including estrogen receptor alpha (ERα), androgen receptor (AR) and progesterone receptor (PR) (Watson et al., 1992; Huang and Harlan, 1993; Skinner et al., 2001). GnRH neurons do express estrogen receptor beta (ERß) (Hrabovszky et al., 2007). Mice lacking ERα have loss of normal cyclicity, elevated LH and absent LH surges while mice lacking ERß have normal cycles, normal LH levels, and are fertile. These reproductive phenotypes indicate that ERα is primarily responsible for estradiol negative and positive feedback to GnRH neurons (Krege et al., 1998; Couse et al., 2003). Furthermore, estradiol induces expression of PR which is important for ovarian negative feedback (Romano et al., 1989; Chappell et al., 1997). Neuronal populations that express ERα or PR may communicate to GnRH neurons either directly or indirectly. Neurons that are sensitive to ovarian steroid hormones have been shown to project to GnRH neurons (Simonian et al., 1999; Wintermantel et al., 2006). This afferent network communicates hormone feedback signals to GnRH neurons through fast-synaptic transmission and neuropeptides (Herbison, 2014).

Fast-synaptic transmission through GABA and, to a lesser degree, glutamate are important sources of input to GnRH neurons (Jansen et al., 2003; Pielecka-Fortuna and Moenter, 2010). GnRH neurons have close appositions with GABAergic fibers (Leranth et al., 1985; Cottrell et al., 2006; Sergeeva and Jansen, 2009) and express both $GABA_A$ and $GABA_B$ receptors (Sim et al., 2000; Temple and Wray, 2005; Zhang et al., 2009). GABAergic neurons that express ERα, PR and AR are located in areas of the hypothalamus that are thought to project to GnRH neurons (Flugge et al., 1986; Leranth et al., 1992; Thind and Goldsmith, 1997; Scott et al., 2000). Of note, many neurons in the anteroventral periventricular area (AVPV) and the arcuate nucleus have been shown to express GABA (Horvath et al., 1997; Ovesjo et al., 2001; Hentges et al.,

2004; Ottem et al., 2004). AVPV neurons also express ERα and GABA expression in AVPV neurons is increased at the time of the surge indicating that GABAergic neurons in the AVPV respond to estradiol feedback (Simerly et al., 1990; Ottem et al., 2004). Neurons in the AVPV, including those which express ERα, project to GnRH neurons (Gu and Simerly, 1997; Simonian et al., 1999). GABAergic neurons have thus emerged as one pathway for the ovarian hormone feedback loop to GnRH neurons.

Functional studies have elucidated how ovarian hormone feedback is conveyed in part through GABAergic transmission to GnRH neurons. To examine the relative effects of different hormones, GABAergic PSCs were recorded in GnRH neurons in brain slices from OVX mice given hormone replacements. Progesterone and estradiol combination treatment decreased GABAergic PSC frequency and amplitude compared to estradiol treatment alone. This suggests that progesterone may alter pre and/or post-synaptic GABAergic signaling to GnRH neurons (Sullivan and Moenter, 2005). In support of the latter, the progesterone derivative allopregnanolone is an allosteric agonist of the $GABA_AR$ (Majewska et al., 1990). Application of allopregnanolone decreased PSC amplitude and altered kinetics of GABAergic PSCs in GnRH neurons. (Sullivan and Moenter, 2003). These data suggest that a potential mechanism for hormonal feedback regulation may be through modulation of the GnRH neuron response to GABA as allopregnanolone amplifies $GABA_AR$-associated current in GnRH neurons from rats (Yin et al., 2008) and increases membrane potential depolarization in response to GABA application (Sim et al., 2001).

In contrast to progesterone, androgen treatment increased GABAergic PSC frequency and amplitude in GnRH neurons (Sullivan and Moenter, 2005). The androgen-mediated increase in GABAergic PSC frequency was activity-independent, suggesting that androgens act to increase synaptic connectivity of GABAergic afferents to GnRH neurons. Androgens may also modulate the postsynaptic current response of GnRH neurons to GABA. The androgen DHEAS allosterically modulates the $GABA_A$ receptor (Liu et al., 2002) and DHEAS treatment decreased GABAergic PSC amplitude in GnRH neurons (Sullivan and Moenter, 2003). Androgens may therefore interfere with progesterone negative feedback through opposing effects on GABAergic signaling. These effects may occur through direct action of ovarian hormones on GABAergic afferents of GnRH neurons and/or on steroid-sensitive populations further upstream.

GnRH neuron response to $GABA_A$ receptor activation

GABA is known as the major inhibitory neurotransmitter in the brain (Curtis and Johnston, 1974) There are many exceptions to this generalized principle, including multiple cell types within the hypothalamus in which GABA has a depolarizing effect on membrane potential (Hewitt et al., 2009; DeFazio et al., 2014). $GABA_A$ receptors are ligand-gated ion channels that are primarily selective for chloride, although there is some permeability for other anions including bicarbonate (Kaila et al., 1989; Sigel and Steinmann, 2012). The direction and magnitude of the electrochemical driving force for chloride depends on the relative intracellular chloride concentration and the membrane potential. The potential at which there is no net driving force for chloride, and thus no net current, is known as the reversal or equilibrium potential. When $GABA_A$ receptors are activated, chloride ions will move down their electrochemical gradient, moving the cell membrane potential towards the $GABA_A$ reversal potential (Bevan et

al., 2000). The neuronal response to GABA therefore depends on the relative intracellular chloride concentration as determined by the relative expression and activity of chloride cotransporters. The chloride co-transporters Na-K-Cl (NKCC1) and K-Cl (KCC2) increase and decrease intracellular chloride, respectively (DeFazio et al., 2000). Many neuron types undergo a switch during development in their response to GABA through decreasing NKCC1 expression and increasing KCC2 expression (Rivera et al., 1999; Ben-Ari, 2002). GnRH neurons maintain elevated intracellular chloride concentrations in adulthood through their relatively increased expression of NKCC1 (DeFazio et al., 2002). The GABA equilibrium potential is depolarized from the average GnRH neuron resting potential, however inhibition of NKCC1 by the antagonist bumetanide results in relative hyperpolarization of the GABA equilibrium potential (DeFazio et al., 2002; Nakane and Oka, 2010).

GnRH neurons from adult female mice, as well as other species including rats and fish, are depolarized by $GABA_A$ receptor activation and are excited to fire action potentials in response to GABA (DeFazio et al., 2002; Watanabe et al., 2009; Nakane and Oka, 2010). Furthermore, activation of $GABA_AR$ evokes an increase in intracellular calcium, a marker of cellular activity, in GnRH neurons (Constantin et al., 2010). Conversely, the $GABA_AR$ antagonist bicuculline has been shown to decrease GnRH neuron firing rate (Moenter and DeFazio, 2005). This experiment was performed in mouse brain slices that were first treated with ionotropic glutamate receptor blockers to mitigate the disinhibiting effects of blocking $GABA_AR$ in GnRH neuron afferents.

Prenatal androgenization (PNA)

Animal models have been used to gain insight into the mechanisms of neuroendocrine dysfunction in PCOS that cannot be studied in women. Various aspects of the PCOS phenotype have been recapitulated through prenatal exposure to elevated androgens (PNA) through maternal androgen treatment in several species including mice, rats, rhesus macaques and sheep (Birch et al., 2003; Sullivan and Moenter, 2004; Foecking et al., 2005; Abbott et al., 2008). The mechanism(s) of prenatal androgen effects on reproductive function is unclear as androgens may act on the mother, placenta and/or enter fetal circulation. Of interest in this regard, PNA rhesus monkey fetuses had elevated testosterone levels following maternal testosterone treatment despite high aromatase expression in the placenta, indicating that the fetus is directly exposed to androgen (Abbott et al., 2008). Testosterone levels in the umbilical veins of female newborns of mothers with PCOS were increased compared to levels in female newborns of control mothers (Barry et al., 2010). Furthermore, global heterozygous and homozygous androgen receptor knock out in female PNA mice were sufficient to protect against development of the PNA phenotype suggesting that these changes are dependent on androgen actions on the PNA fetus (Caldwell et al., 2015).

Adult female PNA mice have impaired reproductive function as evidenced by irregular estrous cycles, reduced number of corpora lutea, and reduced litter size/fertility (Sullivan and Moenter, 2004; Witham et al., 2012; Moore et al., 2013; Caldwell et al., 2014). These reproductive features are suggestive of ovulatory impairment similar to that seen in women with PCOS. PNA mice also show early onset of vaginal opening, an outward marker of female puberty, which may relate to the observation of reported early menarche in women with PCOS (Carroll et al., 2012;

Witham et al., 2012; Dulka and Moenter, 2016). Adult PNA mice also exhibit the hormonal changes commonly seen in women with PCOS including elevated testosterone and LH levels as well as relative glucose intolerance (Sullivan and Moenter, 2004; Moore et al., 2013).

PNA animals have been used to examine the potential role of impaired ovarian hormone feedback in PCOS. Female PNA mice have a diminished LH increase following OVX compared to controls (Moore et al., 2013). Furthermore, OVX PNA mice had less suppression of LH in response to estradiol treatment compared to control OVX mice indicating impaired negative feedback (Moore et al., 2013). In contrast to negative feedback, the effect of PNA on positive feedback is less clear. OVX PNA mice and sheep appear to have typical estradiol-induced LH surges, suggesting that PNA does not alter estradiol positive feedback (Wood et al., 1995; Moore et al., 2013). However, sheep treated with prenatal androgens for days 30-86 of gestation, rather than days 65-86 as was done previously, did not have an estradiol-induced LH surge (Wood et al., 1995; Herbosa et al., 1996). OVX PNA sheep had a dramatically reduced proportion of GnRH neurons that expressed cFos, a protein used as a marker for neuronal activity, in response to an estradiol-induced surge protocol compared to controls, indicating loss of estradiol positive feedback (Wood et al., 1996). PNA may also interfere with progesterone feedback sensitivity, which is impaired in women with PCOS (Pastor et al., 1998; Levine et al., 2005; Veiga-Lopez et al., 2008). Progesterone implants decreased LH pulse frequency in control OVX ewes that were treated with estradiol. In contrast, there was no effect of progesterone on LH pulse frequency or amplitude in PNA OVX ewes treated with estradiol. (Robinson et al., 1999). Furthermore, the estradiol-induced increase in progesterone receptor expression in the hypothalamus was blunted in female PNA compared to control rats (Foecking et al., 2005). As hormone feedback is

communicated to GnRH neurons through their afferent network, the mechanism(s) of impaired hormone feedback in PNA mice may involve this network.

PNA may induce functional and/or organizational changes in the GnRH neuronal network. GnRH neuron firing rate is increased and quiescent periods are decreased in PNA mice compared to diestrus controls (Roland and Moenter, 2011). The increase in GnRH neuron firing rate in PNA mice may be due, at least in part, to PNA-induced alterations in the afferent networks responsible for ovarian feedback, including GABAergic neurons. In line with this potential mechanism, GABAergic transmission frequency and amplitude to GnRH neurons is increased in adult female PNA mice (Sullivan and Moenter, 2004). Endogenous GABA can excite GnRH neurons from adult PNA female mice (Moenter and DeFazio, 2005), thus increased GABAergic transmission to GnRH neurons may contribute to the increased GnRH neuron firing seen in adult PNA mice. This increase in GABAergic transmission frequency is activity-independent, suggesting that it arises due to increased synaptic connectivity rather than presynaptic activation. PNA mice and sheep also show an increase in GnRH neuron spine density, providing further evidence that synaptic connectivity is altered by PNA (Kim et al., 1999; Moore et al., 2013). In adult PNA mice, short and long-term flutamide treatment was sufficient to reverse increases in GABAergic transmission frequency and amplitude (Sullivan and Moenter, 2004) suggesting that changes in the GnRH neuronal network are maintained at least in part through increased androgen levels in adults.

Androgens during development

PCOS may arise from organizational changes to neuroendocrine circuits during prenatal development as well as activational effects of androgens during pubertal and/or adulthood (Abbott et al., 2005). During perinatal development, androgens and estrogens aromatized from androgens act to masculinize the formation and organization of neuronal networks (MacLusky and Naftolin, 1981). In adult mice, androgens have been shown to increase synaptic spines and transmission and may have a similar effect during development (Brawer et al., 1983; Garcia-Segura et al., 1994; Hajszan et al., 2007; Hajszan et al., 2008). In mice, females express AR in the hypothalamus during late prenatal and early postnatal development (Mogi et al., 2015). Female adolescent and adult mice treated with androgens at levels that replicate anabolic steroid overuse showed decreased GABAergic transmission to GnRH neurons (Penatti et al., 2005; Penatti et al., 2011). These findings suggest that programming effects of androgens may underlie changes in the GnRH neuronal network seen in adult PNA mice. GABAergic synaptic organization is altered in adult PNA mice and GABAergic transmission to GnRH neurons is sensitive to androgens making this network a possible substrate for the effects of androgens on pre- and peripubertal neuroendocrine function (Sullivan and Moenter, 2003; Sullivan and Moenter, 2004; Moore et al., 2013).

Developmental origin of PCOS

The importance of the internal and external environment during development for shaping adult function has long been recognized and represents an important framework for understanding health and disease. Prepubertal girls with hyperandrogenemia are at increased risk of developing PCOS as adults (Knudsen et al., 2010). Obese girls are a useful group in which to study the

effects of prepubertal hyperandrogenemia because they have elevated testosterone levels compared to normal weight girls starting as early as Tanner stage 1 and continuing through puberty (Burt Solorzano et al., 2010). Hyperandrogenemia appears to be predominantly adrenal in these girls in contrast to adult women with PCOS in whom excess androgens come predominantly from the ovaries (Rosenfield et al., 2000). Of interest in this regard, girls with premature adrenarche are more likely to develop PCOS (Ibanez et al., 1998; Kousta, 2006).

In addition to hyperandrogenemia, other features of adult women with PCOS, including elevated LH pulse frequency, can emerge during the pubertal transition (Tanner stages 3-4) (Blank et al., 2006; Shayya and Chang, 2010; Collins et al., 2014). During the pubertal transition, girls typically progress from having increased nocturnal LH pulse frequency and amplitude (Wennink et al., 1990) to having greater daytime than nighttime LH levels. Premenarchal girls with hyperandrogenemia (HA) develop the mature pattern of LH pulses at younger ages than girls without HA (Apter et al., 1994). When compared to non-obese girls at the same Tanner stage, obese early pubertal girls did not show an overnight increased in LH amplitude but instead had elevated LH frequency at all times of day (McCartney et al., 2009). Interestingly, further studies showed that obese postmenarchal girls had elevated LH pulse frequency but that blunted day-night changes in LH pulse frequency was seen only in those with hyperandrogenemia (Collins et al., 2014). Furthermore, the high LH pulse frequency in hyperandrogenemic girls was resistant to suppression by progesterone, similarly to women with PCOS (Chhabra et al., 2005; Collins JS, 2013). However, it is not known if excess androgens during adolescence may be interfering with progesterone feedback in a similar manner as in adult women with PCOS. These studies suggest that hyperandrogenemia during peripubertal development leads to altered neuroendocrine

function. However, it is not known when or how neuroendocrine changes may arise relative to pubertal development. If any changes exist, they may represent an important pathological step towards impaired negative feedback, overactive GnRH/LH pulse frequency and downstream ovarian androgen production characteristic of women with PCOS. Hyperandrogenemia may thus be both a cause and a consequence in a vicious cycle of neuroendocrine dysfunction in prepubertal girls and adult women with PCOS.

The following chapter will examine the hypotheses that direct GABAergic transmission to GnRH neurons develops prepubertally in both sexes and that PNA alters the synaptic organization of this network prior to adult manifestations of reproductive dysfunction in females. We will further begin to characterize the response of GnRH neurons to GABA during prepubertal development and functional changes induced by PNA. Finally, we will discuss the implications and limitations of our findings and propose interesting avenues for future research.

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Chapter 2 Prepubertal development of GABAergic transmission to gonadotropin releasing hormone (GnRH) neurons and postsynaptic response are altered by prenatal androgenization

Significance Statement

The central neuronal network that regulates reproduction is overactive in polycystic ovary syndrome [PCOS), a leading cause of infertility. Recent evidence of neuroendocrine dysfunction in mid-pubertal girls suggests the pathophysiological mechanisms underlying PCOS may arise before pubertal maturation. Prenatal exposure to androgens (PNA) in mice mimics several neuroendocrine features of PCOS. GABAergic transmission to GnRH neurons is important for reproduction and is increased in adult PNA mice. Neither the typical development of this network nor when changes with PNA and sex arise relative to puberty are understood. These studies provide evidence that PNA alters prepubertal development of the GABAergic network afferent to GnRH neurons, including both the presynaptic organization and postsynaptic response. These changes may contribute to reproductive dysfunction in adults.

Abstract

GnRH neurons regulate reproduction through pulsatile GnRH release. Women with polycystic ovary syndrome have persistently elevated LH release frequency reflecting GnRH release; this exacerbates hyperandrogenemia and disrupted reproductive cycles characteristic of this disorder. Clinical evidence suggests neuroendocrine features of PCOS may manifest peripubertally. Adult mice prenatally exposed to androgens (PNA) mimic several reproductive features of PCOS.

GnRH neurons from these mice have increased firing activity and receive increased excitatory GABAergic transmission. When these changes emerge during development is unknown. To study typical postnatal development of GABAergic transmission and effects of PNA treatment and sex, whole-cell voltage-clamp recordings were made of GABAergic postsynaptic currents (PSCs) in GnRH neurons in brain slices from prepubertal through adult control and PNA female and male mice. GABAergic transmission was present by one-week of age in females and males and increased in frequency, reaching adult levels at three and four weeks, respectively. GABAergic PSC frequency was elevated in three-week-old PNA vs control females. PSC frequency in both controls and PNA mice was activity-independent, suggesting PNA induces changes in synapse organization. PNA also alters functional response of GnRH neurons to GABA. GABA induced firing in fewer neurons from three-week-old PNA than control females; membrane potential depolarization induced by GABA was also reduced in cells from PNA mice at this age. PNA thus induces changes during development in the presynaptic organization of the GABAergic network afferent to GnRH neurons as well as the postsynaptic GnRH neuron response, both of which may contribute to adult reproductive dysfunction.

Introduction

GnRH neurons form the final common pathway for central regulation of reproduction. GnRH is released in pulses (Clarke and Cummins, 1982; Moenter et al., 1992). During the typical female reproductive cycle, shifts from low to high frequency GnRH release help drive differential synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary (Wildt et al., 1981; Haisenleder et al., 1991). FSH and LH regulate ovarian follicle maturation and steroidogenesis (Baird et al., 1976; Zeleznik and Fairchild-Benyo, 1994).

Steroid feedback in turn modulates GnRH/LH release (Leipheimer et al., 1984; Nippoldt et al., 1989; Moenter et al., 1990; Tilbrook et al., 1991; McCartney et al., 2002). GnRH neurons do not express detectable levels of most steroid hormone receptors (Watson et al., 1992; Huang and Harlan, 1993; Skinner et al., 2001); therefore these signals are likely conveyed via steroid-sensitive presynaptic inputs (Wintermantel et al., 2006; Cheong et al., 2015).

The frequency of LH secretion (a bioassay for GnRH release) and steroid feedback regulation of LH release are altered in women with polycystic ovarian syndrome. PCOS is a leading cause of infertility, conservatively affecting 8% of reproductive-aged women (Goodarzi et al., 2011). Women with PCOS have persistently elevated GnRH/LH release frequency rather than cyclical shifts in pulse frequency (Burt Solorzano et al., 2012; McCartney and Marshall, 2016) and are less sensitive to progesterone negative feedback (Pastor et al., 1998). Increased GnRH release frequency contributes to the pathology of PCOS by altering LH and FSH levels, thus exacerbating elevated ovarian androgen production and irregular menstrual cycles characteristic of this disorder. Manifestations of nascent PCOS are increasingly being detected at younger ages; for example, evidence of elevated LH pulse frequency and hyperandrogenemia may be see in mid-pubertal girls (Tanner stages 3-4) (Blank et al., 2006; Shayya and Chang, 2010; Collins et al., 2014). These clinical findings suggest that antecedents of PCOS may arise during or even before pubertal development.

Exposure to androgens during prenatal development produces neuroendocrine features similar to those of PCOS in adult animals, including rodents, sheep and rhesus macaques (Birch et al., 2003; Sullivan and Moenter, 2004; Abbott et al., 2005; Foecking et al., 2005). Adult female

prenatally androgenized (PNA) mice have elevated LH pulse frequency, increased testosterone levels and disrupted estrous cycles (Sullivan and Moenter, 2004; Witham et al., 2012; Moore et al., 2015). GnRH neurons in brain slices from these mice have a higher firing rate (Roland and Moenter, 2011) and receive higher frequency GABAergic transmission (Sullivan and Moenter, 2004; Roland and Moenter, 2011). GnRH neurons from control mice maintain high intracellular chloride even in adulthood and thus exhibit an excitatory response to GABA action via $GABA_A$ receptors ($GABA_A$Rs) (DeFazio et al., 2002; Herbison and Moenter, 2011). Additionally, GABAergic fiber appositions to GnRH are increased in adult PNA mice (Moore et al., 2015). GABAergic afferents are important mediators of steroid feedback regulation of GnRH neuron function (Petersen et al., 2003; Sullivan and Moenter, 2005; Christian and Moenter, 2007; Pielecka-Fortuna and Moenter, 2010). Increases in GABAergic transmission could thus contribute to the increased GnRH neuron activity in the adult PNA mouse.

Despite the importance of GABAergic afferents in the regulation of GnRH neurons, little is known about either the typical rates of GABAergic transmission across the pubertal transition or when differences attributable to sex or PNA exposure emerge. The prepubertal period and pubertal transition may be critical periods during which alterations in synaptic organization and/or function may have broad implications for adult reproductive physiology. We examined GABAergic synaptic transmission to GnRH neurons and GnRH neuron response to GABA in brain slices from prepubertal through adult female and male mice to understand the typical development of this network and the functional changes induced by PNA.

Materials and Methods

All reagents were purchased from Sigma-Aldrich unless noted.

Animals: GnRH-GFP B6 mice (Suter et al., 2000) were bred in our colony. All mice were provided with water and Harlan 2916 chow *ad libitum* and were held on a 14L:10D light cycle with lights on at 0400 Eastern Standard Time. To generate PNA mice, a GnRH-GFP and a CD1 female were paired for 1-3 weeks 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 days 16–18 of gestation (day 1, copulatory plug observed). Controls (con) included mice from vehicle-treated or uninjected GnRH-GFP dams because in previous studies (Roland and Moenter, 2011), no differences were observed between these groups and they were combined for analysis. The CD1 mouse provided maternal and nutritional support that our observations indicate increase survival of PNA pups. Litter sizes were adjusted to <15 pups by culling CD1 pups to normalize nutrition. Pups not used for recordings were weaned at three weeks of age. Female and male mice at postnatal ages 1, 2, 3, 4 weeks ± 1 day and adults (42-120 days old) were used for recording experiments. Experiments on female mice that had attained puberty were done on diestrus. The Institutional Animal Care and Use Committee of the University of Michigan approved all procedures.

Brain slice preparation: All solutions were bubbled with 95% O2/5% CO2 throughout the experiments and for at least 15 minutes before exposure to tissue. Brain slices were prepared through the preoptic area as previously described (DeFazio et al., 2002; Chu and Moenter, 2005; Glanowska and Moenter, 2015; Ruka et al., 2016). Briefly, brains were rapidly removed and placed in ice-cold sucrose saline containing (in mM): 250 sucrose, 3.5 KCL, 26 $NaHCO_3$, 10

glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal slices (300 μm) were made using a LeicaVT12000S vibratome (Leica Biosystems). Slices were incubated at room temperature for 30 minutes in a 50-50 solution of sucrose saline and ACSF containing (in mM): 135 NaCl, 3.5 KCL, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 310 mOsm/L, pH 7.4. Slices were then transferred to 100% ACSF at room temperature for 0.5-5 h before recording. Slices were used within 6 hours of preparation.

Electrophysiology recording and data acquisition: Slices were transferred to a recording chamber and perfused with oxygenated ACSF at a rate of 3 mL/min at 30±1ºC. ACSF contained 20 μM CNQX and 20 μM APV (Tocris Bioscience) to block ionotropic glutamate receptors. When ionotropic receptors for both glutamate and GABA are blocked, no fast-synaptic transmission is detected in GnRH neurons, suggesting that these inputs account for the vast majority of fast synaptic transmission (Sullivan et al., 2003). Recording pipettes were pulled from borosilicate glass pipettes (World Precision Instruments, 1.65/1.1 mm OD/ID) using a P-97 puller (Sutter Instruments). Pipettes were filled with a solution containing 140 mM KCl, 10 mM HEPES, 5 mM EGTA, 0.1 mM CaCl2, 4 mM MgATP and 0.4 mM NaGTP, pH 7.2, 310 mOsm/L. Pipettes with resistances of 2-4 MΩ when filled with this pipette solution were used. All voltages reported were corrected for liquid junction potential during analysis. GnRH-GFP neurons were identified by brief illumination with 470 nm light using an Olympus BX51WI microscope. Recordings were made using one channel of an EPC-10 dual patch-clamp amplifier and Patchmaster acquisition software (Heka Elektronic) running on a Macintosh G4 computer. Membrane currents were filtered at 6.5 kHz and digitized at 10 kHz. Cell location was noted and mapped to an atlas to determine if cell location-based differences emerged (Paxinos and

Franklin, 2001). No differences in response attributable to rostral-caudal and medial-lateral distribution were evident. No more than three cells were recorded per animal. The variability between GnRH neurons recorded from a single mouse was similar to the variability of GnRH neurons among mice.

GABAergic postsynaptic current (PSC) recordings: Whole-cell voltage-clamp recordings were made following formation of a high resistance seal (≥1.3 GΩ) between the pipette tip and cell membrane. Spontaneous GABAergic PSCs (sPSCs) were recorded at -55 mV. In some cells, activity-independent miniature PSCs (mPSCs) were recorded in the presence of 1μM TTX following measurement of sPSCs. To monitor cell health and recording quality, input resistance (>500 MΩ), series resistance (<20 MΩ uncompensated, <20% change during the analyzed recording period), holding current (-60 pA to +5 pA) and membrane capacitance (stable) were measured every 2 min from the average of 16 traces recorded in response to a 5 mV hyperpolarizing step; traces with synaptic activity were excluded from analyses.

GABA application: Local pressure application of GABA was accomplished as described (DeFazio et al., 2014). Pipettes were filled with 100 μM GABA in a HEPES-buffered saline solution containing (in mM): 150 NaCl, 10 HEPES, 10 glucose, 3.5 KCl, 2.5 CaCl2, 1.3 MgCl2, pH 7.2 with NaOH. This pipette was placed ~15 μM from the soma, and a pulse of 5-10 psi was delivered using a PV820 picospritzer (World Precision Instruments, Inc.). To determine if GABA induces firing activity in GnRH neurons, on-cell voltage-clamp recordings were made and the response to GABA was evaluated during a 150 ms window after rapid GABA

application. This was repeated three times per cell with ≤30 s between measurements. Pressure application of the HEPES-buffered solution without GABA did not induce firing (data not shown).

To assess the membrane potential response to rapid GABA application, membrane potentials were measured in the on-cell configuration as described (Fricker et al., 1999; Verheugen et al., 1999; DeFazio et al., 2014). Briefly, membrane potential is calculated by measuring potassium current in response to a voltage ramp from -100 to +200 mV over 50 msec. This method assumes that the intracellular concentration of potassium is similar to that in the pipette solution, resulting in a reversal potential for potassium (E_K) across the patch near 0 mV. Under these conditions, the only driving force for potassium current is the potential difference across the patched cell membrane, thus the ramp potential at which the leak-corrected potassium current is 0 pA reveals the membrane potential of the cell. Repeating this procedure twice at 100 ms intervals allows baseline membrane potential to be estimated, a puff of GABA applied locally, and membrane potential in response to GABA estimated. This entire process was repeated 4-12 times per cell with ≥30 s between measurements. The average of the measurements is reported for each cell.

Gramicidin perforated-patch recordings: Gramicidin perforated-patch recordings were performed as in (DeFazio et al., 2002) with minor modifications. Gramicidin stock solutions (50 mg/mL in DMSO) were prepared weekly, protected from light and kept at 4^{o}C. Working concentration (50-100 μg/mL) was made fresh for each cell in pipette solution and sonicated for 10-20 seconds immediately before use. Patch pipettes (2−4.5 MΩ) were back filled with 1.5-2.5 μl of gramicidin-free pipette solution followed by gramicidin solution. Two pipette solutions

were used, one with high chloride and one with low chloride; values obtained with these two solutions will be similar if inadvertent patch rupture has not occurred. Low chloride pipette solution consisted of (in mM): 139 mM K gluconate, 1 mM KCl, 10 mM HEPES, 5 mM EGTA, 0.1 mM CaCl2, 4 mM MgATP and 0.4 mM NaGTP, pH 7.2, 310 mOsm/L. For high chloride pipette solution, K gluconate was replaced with KCl. Membrane potential for recordings with 1 mM or 140 mM chloride were corrected for liquid junction potentials of 16.5 mV and 4.9 mV, respectively.

After obtaining a minimum of 1 GΩ resistance seal, 5 mV hyperpolarizing steps from a holding potential of -65 mV were made in voltage-clamp to monitor membrane perforation. Recordings were initiated when the series resistance fell below 50 MΩ. Voltage steps (-80 mV to +5 mV, 15 mV interval, 500 ms) were paired with local GABA application as described above to determine the reversal potential; GABA was applied 300 ms into the 500 ms voltage step to allow the membrane current to stabilize before drug application. A 5 mV hyperpolarizing step was given before the start of each voltage step to determine series resistance; each step potential was independently corrected for voltage error resulting from series resistance in the analysis. Voltage steps and accompanying GABA application were delivered at 30 s intervals to ensure that applied GABA was cleared between measurements. Baseline current was measured just before GABA application, and GABA response was determined as the peak current following GABA application. The reversal potential was determined from the crossing of the current-voltage relationship of the baseline current and the GABA response using a custom-written software in IGOR Pro and was verified by eye for each recording. Only reversals that were stable (<5 mV variation) for at least two complete measurements were accepted. Rupture of perforated-patch

recordings to whole-cell mode was detected by an abrupt decrease in access resistance, increase in membrane capacitance, broadening on capacitive transient shape and rapid change in the measured reversal potential (hyperpolarized with 1 mM chloride pipette solution, depolarized with 140 mM chloride pipette solution). Any recordings in which any of these changes was detected were discarded.

Analysis: PSC data were analyzed using custom software (Sullivan et al., 2003; DeFazio et al., 2014) written in IgorPro (Wavemetrics). Every PSC was visually confirmed. Frequency is the total number of events detected divided by the duration of the recording. Amplitude, rise time, decay and full-width half maximum (FWHM) are reported as the mean ± SEM of all events in a recording. 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: Data are reported at mean ± SEM. Data were examined for normal distribution using the Shapiro-Wilk test. Statistical analyses were performed (GraphPad Prism) with tests dictated by data distribution and experimental design; specific tests are indicated in the figure legends and tables. For age and sex comparisons, two-way ANOVA followed by Fisher's least significant difference *post hoc* was used; this choice was justified by the large number of comparisons made (i.e., 45 different comparisons between either sex or treatment at five different ages). $p<0.05$ was accepted as significant but all p values <0.1 are reported.

Results

GABAergic transmission to GnRH neurons occurs before puberty in females and males. Whole-cell voltage-clamp recordings of spontaneous GABAergic PSC frequency in GnRH neurons were made in brain slices from 1, 2, 3 and 4-week old and adult female and male control and PNA mice (number of cells per group shown in Table 1). There were no differences in passive properties except for capacitance which was increased with age but was not altered by sex or PNA treatment. These changes would not be expected to affect the data reported. (Table 2, 3). Representative recordings are shown in Figure 1, group comparisons in Figure 2 and Table 4. Low frequency GABAergic transmission was observed at one week of age in all cells studied. In cells from control females, GABAergic transmission frequency increased between two and three weeks of age ($p<0.05$, Figure 2A). In cells from control males, an increase was not observed until four weeks of age ($p<0.05$). PSC frequency was higher in males than females at four weeks of age ($p<0.05$). Androgenization of PNA mice was confirmed in surviving female littermates by younger age of vaginal opening (32.9±0.6 days old n=20 con vs 28.7±0.4 days old PNA n=21, $p<0.05$), disrupted estrous cycles (time spent in estrus, 23% con n=12 vs 8% PNA n=12 $p<0.05$) and greater anal-genital distance (5.5±0.2 mm con n=12 vs 6.6±0.3 PNA n=12 $p<0.05$).

Table 1. Cells/group for sPSC recordings.

age (wks)	**CON ♀**	**PNA ♀**	**CON ♂**	**PNA ♂**
1	7	13	6	5
2	13	8	7	5
3	11	12	13	9
4	11	15	11	10
>6	9	13	6	10

Table 2. Membrane properties of GnRH neurons. Values are mean±SEM

Group	age (wks)	Input resistance (MΩ)	Series resistance (MΩ)	Membrane Capacitance (pF)	Holding current (pA)
♀ CON	1	1205±265	16.9±4.1	9.6±2.9	-33.9±8.4
	2	1063±541	14.2±2.7	12.5±2.8	-42.5±15.3
	3	1022±236	15.5±3.7	12.5±3.0	-36.8±16.2
	4	1193±336	16.7±4.7	12.2±3.9	-34.4±16.6
	>6	1263±354	16.5±4.8	13.3±2.9	-32.0±15.0
♀ PNA	1	1181±345	16.6±3.2	10.8±4.7 *a*	-41.5±12.9
	2	1166±340	14.5±3.6	13.5±6.2	-35.9±15.0
	3	1147±436	14.9±4.5	12.1±4.4	-38.8±14.2
	4	1135±303	13.9±4.1	13.3±2.3 *b*	-31.7±11.2
	>6	1203±308	16.0±5.1	14.6±2.1 *b*	-35.9±18.2
♂ CON	1	1293±279	17.4±6.3	14.1±9.2 *a*	-35.7±12.1
	2	946±279	15.6±2.1	10.2±1.0 *a*	-44.7±13.3
	3	1127±282	16.2±3.3	12.5±3.3 *a*	-31.5±9.2
	4	989±279	14.6±2.3	12.2±2.7 *a*	-43.0±20.8
	>6	851±184	15.1±4.8	16.7±2.9 *b*	-48.0±26.4
♂ PNA	1	1180±312	16.0±3.5	8.4±2.7 *a*	-41.8±20.7
	2	939±242	16.1±4.9	15.0±8.8 *b*	-46.5±6.9
	3	921±330	13.4±4.8	13.3±6.5 *b*	-45.9±12.9
	4	942±272	15.5±4.4	12.7±3.3	-41.0±13.0
	>6	1146±472	15.1±4.6	13.8±3.4 *b*	-33.4±17.6

Different lower case letters indicate differences with age within a group. Significant differences defined as $p<0.05$. Values without letters are not different from any other age, sex or treatment.

Table 3. Two-way ANOVA parameters for comparison of membrane properties among groups

Groups	Parameter	Sex	Age	Interaction
Con ♀ vs Con ♂	Input resistance	F(1, 84)=1.72	F(4, 84)=0.82	F(4, 84)=1.56
	Series resistance	F(1, 84)= 0.03	F (4, 84)=0.42	F (4, 84)=0.71
	Capacitance	F(1, 84)=0.26	F(4, 84)=4.66**	F(4, 84)=1.92
	Holding current	F(1, 84)=0.97	F(4, 84)=0.14	F(4, 84)=0.93
PNA ♀ vs Con ♂	Input resistance	F(1, 94)=3.36	F(4, 94)=1.02	F(4, 94)=0.92
	Series resistance	F(1, 94)=1.52	F(4, 94)=0.83	F(4, 94)=0.81
	Capacitance	F(1, 94)=0.07	F(4, 94)=2.53*	F(4, 94)=0.29
	Holding current	F(1, 94)=0.03	F(4, 94)=1.05	F(4, 94)=0.99
Groups	**Parameter**	**Treatment**	**Age**	**Interaction**
Con ♀ vs PNA ♀	Input resistance	F(1, 102)=0.07	F(4, 102)=0.49	F(4, 102)=0.73
	Series resistance	F(1, 102)=1.76	F(4, 102)=0.44	F(4, 102)=0.70
	Capacitance	F(1, 102)=0.03	F(4, 102)=2.38	F(4, 102)=0.51
	Holding current	F(1, 102)=1.13	F(4, 102)=0.86	F(4, 102)=0.32
Con ♂ vs PNA ♂	Input resistance	F(1, 73)=0.11	F(4, 73)=2.02	F(4, 73)=0.91
	Series resistance	F(1, 73)=1.47	F(4, 73)=0.41	F(4, 73)=1.81
	Capacitance	F(1, 73)=0.01	F(4, 73)=2.71*	F(4, 73)=1.96
	Holding current	F(1, 73)=1.58	F(4, 73)=1.92	F(4, 73)=0.53

*p<0.05; **p<0.01

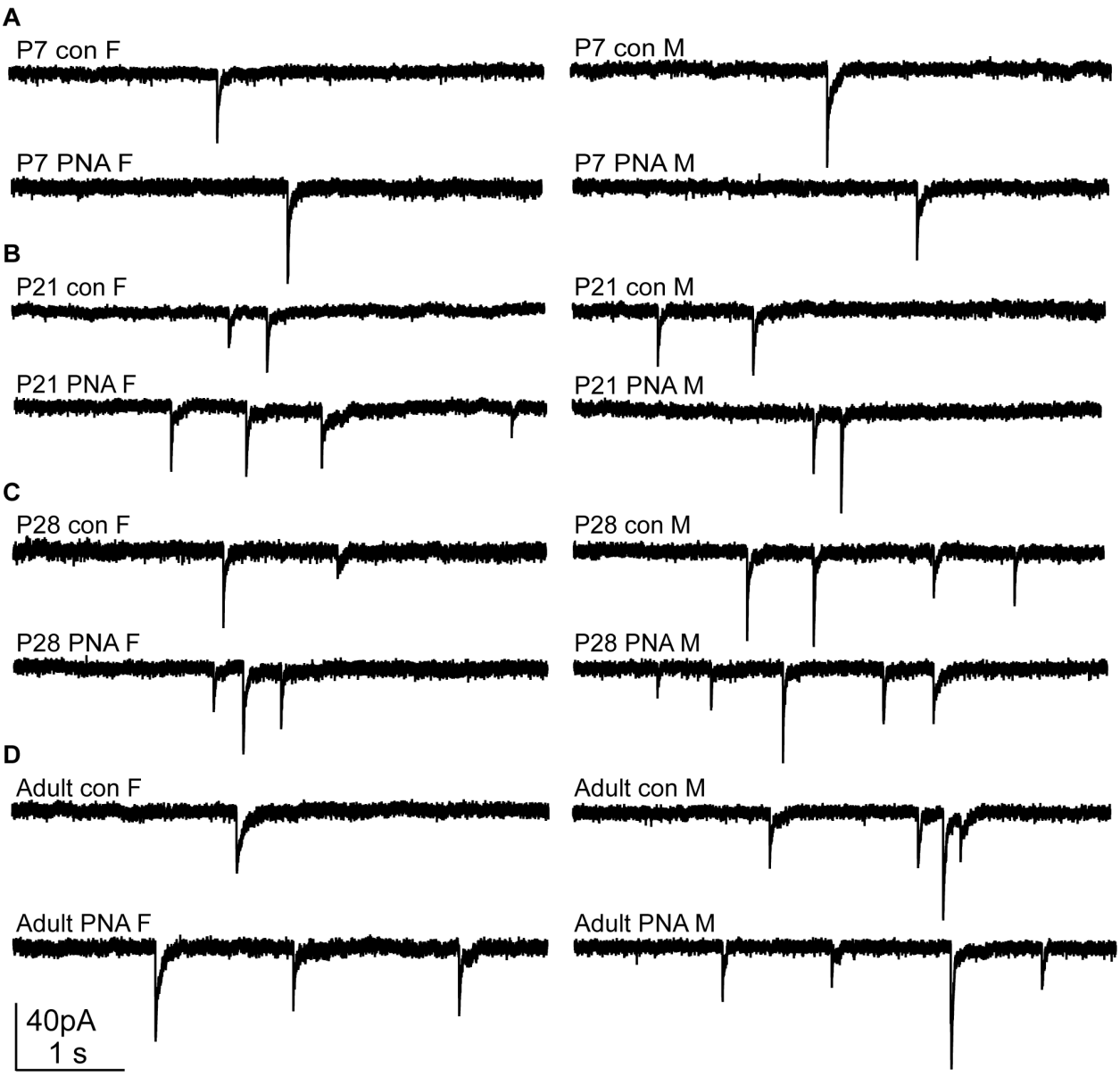

Figure 1. Representative recordings of GABAergic PSCs in GnRH neurons in female (F, left) and male (M, right) control (top) and PNA (bottom) mice at ages **A**, one week; **B** three weeks; **C**, four weeks; **D**, adults >6 weeks.

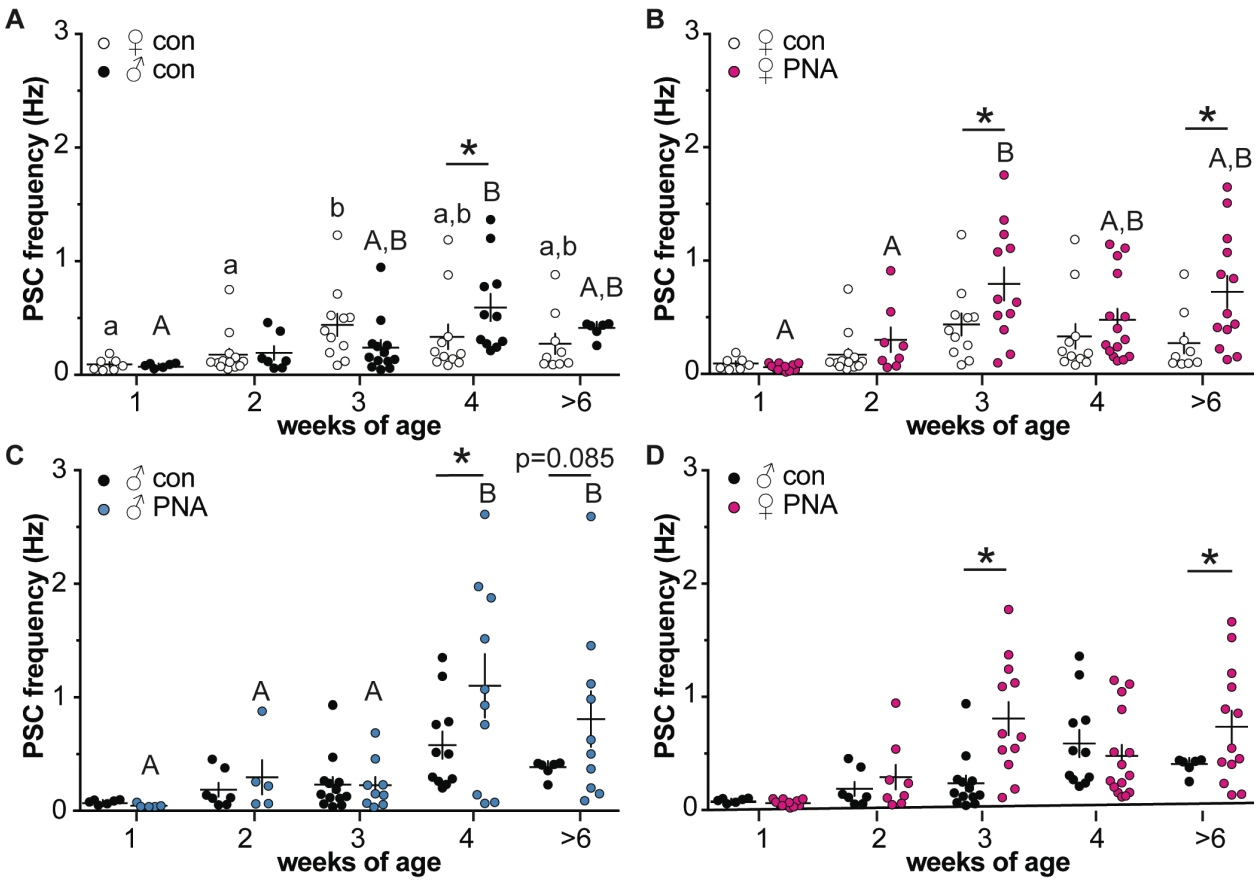

Figure 2. Spontaneous GABAergic transmission frequency to GnRH neurons changes throughout the prepubertal period in both sexes and is altered by PNA. A-D, Individual values and mean ± SEM of GABAergic PSC frequency at 1, 2, 3, 4 weeks and adults >6 weeks. A, control females (open circles) and males (black circles), two-way ANOVA/Fisher's LSD B, control and PNA (magenta circles) females, two-way ANOVA/Fisher's LSD. C, control and PNA (blue circles) males, two-way ANOVA/Fisher's LSD. D, PNA females and control males, two-way ANOVA/Fisher's LSD. Different letters of the same case indicate differences with age within a group; * indicates $p<0.05$ between groups at each age.

Table 4. Two-way ANOVA parameters for comparison of sPSC frequency, amplitude and membrane potential change among groups

Parameter (Figure displaying data)	Sex/Treatment	Age	Interaction
sPSC frequency (2A)	F(1, 84)=1.72	F(4, 84)=5.64***	F(4, 84)=2.20*
sPSC amplitude (3A)	F(1, 84)=0.17	F(4, 84)=1.76	F(4, 84)=1.30
sPSC frequency (2B)	F(1, 102)=9.45**	F(4, 102)=8.44****	F(4, 102)=1.57
sPSC amplitude (3B)	F(1, 102)=0.01	F(4, 102)=0.5	F(4, 102)=0.7
sPSC frequency (2C)	F(1, 73)=3.63*	F(4, 73)=7.77****	F(4, 73)=1.24
sPSC amplitude (3C)	F(1, 73)=0.07	F(4, 73)=3.03*	F(4, 73)=0.88
sPSC frequency (2D)	F(1, 94)=6.37*	F(4, 94)=7.14****	F(4, 94)=3.73**
sPSC amplitude (3D)	F(1, 94)=0.20	F(4, 94)=0.82	F(4, 94)=1.80
mPSC frequency (5A)	F(1, 43)=3.63*	F(3, 43)=2.78*	F(3,43)=1.47
mPSC amplitude (5B)	F(1, 43)=0.37	F(3,43)=0.61	F(3,43)=1.62
Δ membrane potential (5F)	F(1, 56)=2.3	F(3, 56)=2.4	F(3, 56)=2.1

*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

PNA alters prepubertal development of GABAergic transmission to GnRH neurons in females and males. In cells from PNA females, GABAergic transmission frequency increased between two and three weeks of age as in controls, but the increase was more robust in PNA females (Figure 2B). As a result, PSC frequency was elevated in cells from PNA compared to control females at three weeks of age ($p<0.05$); this increase was maintained in adults ($p<0.05$), confirming previous observations (Sullivan and Moenter, 2004). In males, GABAergic transmission frequency was increased at four weeks of age in cells from PNA compared to control males ($p<0.05$), but not in adulthood ($p=0.085$, Figure 2C). To address the question of whether or not GABAergic transmission in PNA females reflects masculinization of the network, we compared PNA females to control males (Figure 2D). Cells from PNA females had higher

GABAergic PSC frequency than cells from control males at three weeks of age ($p<0.05$), and in adults ($p<0.05$). GABAergic transmission in cells from PNA females thus showed a different pattern of prepubertal development from control males. There was no effect of age, sex or treatment on GABAergic PSC amplitude (Figure 3, Table 4). There was no overall trend with age, sex or treatment in sPSC kinetics (Table 5 and 6). FWHM was smaller at four weeks in control females and thus elevated in controls at two weeks of age and in adults.

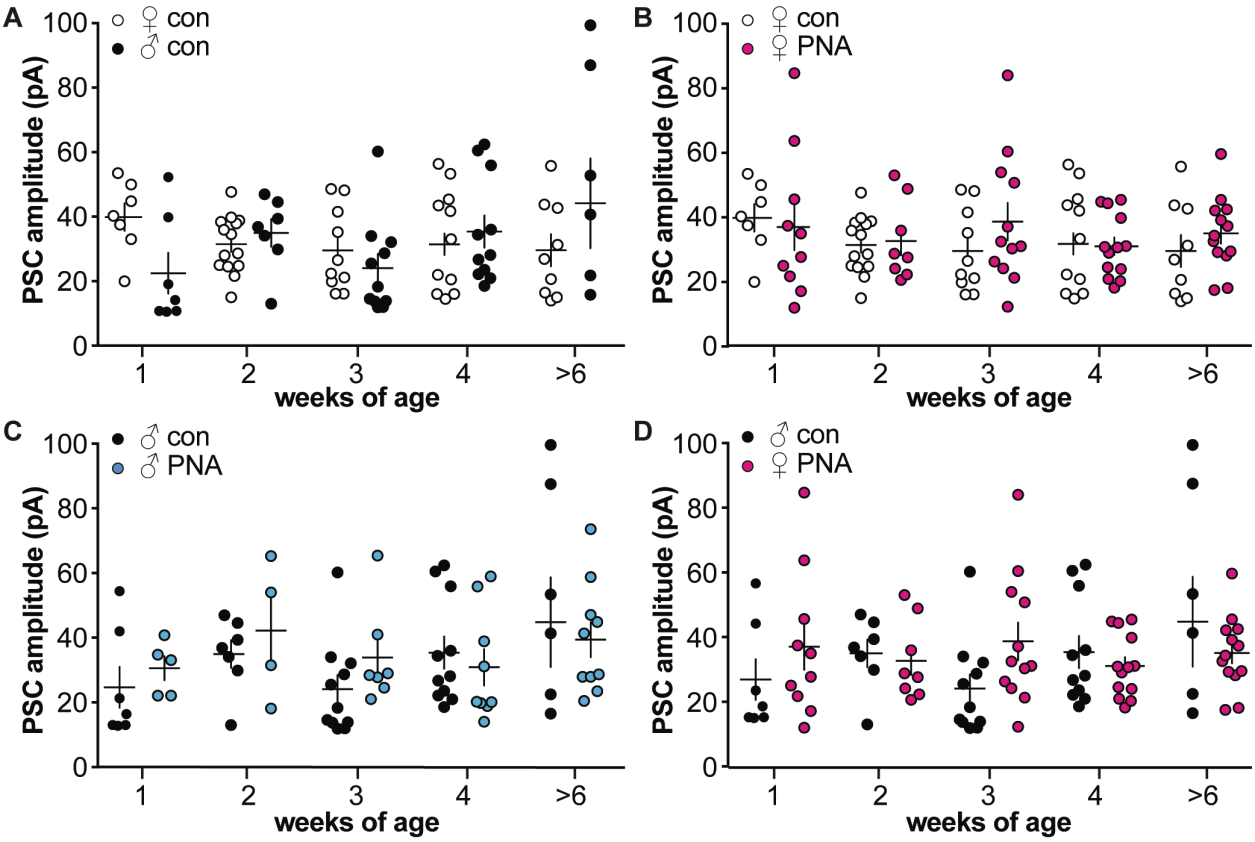

Figure 3 Amplitude of spontaneous GABAergic PSCs is not altered by PNA or sex. **A-D**, Individual values and mean ± SEM (crosses) of GABAergic PSC amplitude at 1, 2, 3, 4 weeks and adults >6 weeks. No differences were seen when analyzed using a two-way ANOVA/Fisher's LSD. **A**, control females (open 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.

Table 5. .Mean±SEM kinetic parameters of GABA sPSCs in GnRH neurons.

Group	age (wks)	rise time (ms)	decay (ms)	FWHM (ms)
	1	0.37±0.06	17.2±2.2	5.99±0.75
	2	0.38±0.06	18.8±1.0	6.72±0.53 *a*
♀ CON	3	0.52±0.08	17.0±1.4	5.42±0.32
	4	0.49±0.09	19.2±1.5	4.17±0.65 *b*
	>6	0.43±0.11	19.4±1.5	6.41±0.64 *a*
	1	0.52±0.17	18.8±3.5	5.94±0.57
	2	0.50±0.10	23.1±2.4	6.69±0.57
♀ PNA	3	0.32±0.07	18.4±1.7	4.99±0.58
	4	0.49±0.05	17.4±1.0	5.92±0.38
	>6	0.42±0.07	20.9±1.0	6.02±0.82
	1	0.62±0.14	22.6±3.2	7.57±0.13
	2	0.40±0.08	20.4±3.0	6.69±0.57
♂ CON	3	0.31±0.10	18.2±2.8	7.73±1.06
	4	0.33±0.09	20.6±2.0	5.92±0.38
	>6	0.22±0.09	20.7±1.5	6.02±0.82
	1	0.35±0.08	23.3±2.8 *a*	6.39±0.61
	2	0.29±0.09	25.2±0.9 *a*	8.30±0.59
♂ PNA	3	0.57±0.05	14.3±2.2 *b*	5.38±0.52
	4	0.48±0.08	20.2±1.1	6.76±0.51
	>6	0.36±0.05	21.9±2.2 *a*	5.99±0.46

Different lower case letters indicate differences with age within a group. Significant differences defined as $p<0.05$. Values without letters are not different from any other age, sex or treatment.

Table 6. Two-way ANOVA parameters for comparison of GABA sPSC kinetics among groups

Groups	Parameter	Sex	Age	Interaction
Con ♀ vs Con ♂	Rise time	F(1, 84)=3.24	F(4, 84)=0.30	F(4, 84)=1.04
	decay	F(1, 84)= 2.86	F (4, 84)=0.60	F (4, 84)=0.33
	FWHM	F(1, 84)=2.10	F(4, 84)=0.22	F(4, 84)=0.23
PNA ♀ vs Con ♂	Rise time	F(1, 94)=4.48*	F(4, 94)=1.40	F(4, 94)=0.40
	decay	F(1, 94)=0.30	F(4, 94)=0.96	F(4, 94)=0.79
	FWHM	F(1, 94)=0.20	F(4, 94)=0.16	F(4, 94)=0.17
Groups	**Parameter**	**Treatment**	**Age**	**Interaction**
Con ♀ vs PNA ♀	Rise time	F (1, 67) = 0.08	F (4, 67) = 0.30	F (4, 67) = 1.29
	decay	F(1, 102)=1.84	F(4, 102)=1.68	F(4, 102)=1.14
	FWHM	F(1, 102)=0.05	F(4, 102)=3.12*	F(4, 102)=0.65
Con ♂ vs PNA ♂	Rise time	F(1, 73)=2.00	F(4, 73)=1.13	F(4, 73)=1.86
	decay	F(1, 73)=0.12	F(4, 73)=2.81*	F(4, 73)=0.90
	FWHM	F(1, 73)=0.30	F(4, 73)=0.40	F(4, 73)=0.40

*$p<0.05$

The frequency of GABAergic transmission to GnRH neurons is activity-independent. Increased frequency of neurotransmission with postnatal development or PNA treatment could result from increased presynaptic activity and/or increased synaptic connectivity to GnRH neurons. To distinguish between these mechanisms, the fast voltage-gated sodium channel blocker tetrodotoxin (TTX) was added during recordings to isolate activity-independent neurotransmission, which is proportional to the number of functional synaptic connections (Auger and Marty, 2000; Kaeser and Regehr, 2014). As with sPSCs, mPSC frequency was increased in adult PNA females compared to controls; in three-week old females, mPSC frequency did not meet criteria for statistical significance ($p=0.104$, Figure 4A, Table 4). Neither amplitude nor kinetics of mPSCs differed with age or PNA treatment (Figure 4B, Table 4, 7 and 8). Similarly, comparison of sPSC and mPSC frequency and amplitude in the same cell revealed no difference with age or PNA treatment (Figure 4C, D, Table 9).

Table 7 Mean±SEM kinetic parameters of GABA mPSCs in GnRH neurons.

Group	age (wks)	rise time (ms)	decay (ms)	FWHM (ms)
♀ **CON**	2	0.41±0.22	22.40±5.06	6.60±0.96
	3	0.31±0.20	20.61±4.04	5.10±0.62
	4	0.49±0.40	22.05±2.67	5.28±0.33
	>6	0.29±0.17	20.63±3.66	6.18±0.99
♀ **PNA**	2	0.60±0.33	17.29±7.28	5.74±0.22
	3	0.50±0.40	18.84±6.54	6.61±0.61
	4	0.38±0.25	20.12±5.59	6.49±0.38
	>6	0.37±0.25	23.17±4.40	7.27±0.55

No differences were observed among groups.

Table 8. Two-way ANOVA parameters for comparison of GABA mPSC kinetics in control vs PNA females.

Groups	Parameter	Treatment	Age	Interaction
Con ♀ vs PNA ♀	Rise time	F(1, 43)=0.341	F(4, 43)=0.923	F(4, 43)=0.206
	decay	F(1, 43)=0.808	F(4, 43)=2.24	F(4, 43)=1.53
	FWHM	F(1, 43)=0.161	F(4, 43)=2.12	F(4, 43)=0.0929

No differences were observed among groups.

Table 9. Two-way repeat-measures ANOVA parameters for comparison of sPSCs and mPSCs

Factor	age (wks)	Treatment	TTX	Interaction	CON n	PNA n
frequency	2	F(1,9)=0.8	F(1,9)=2.3	F(1,9)=1.4	5	6
	3	F(1,10)=1.0	F(1,10)=0.3	F(1,10)=0.08	7	6
	4	F(1,10)=0.5	F(1,10)=2.5	F(1,10)=1.8	6	6
	>6	F(1,9)=4.5	F(1,9)=0.07	F(1,9)=0.15	6	5
amplitude	2	F(1,9)=0.6	F(1,9)=2.9	F(1,9)=1.5	5	6
	3	F(1,10)=0.7	F(1,10)=3.2	F(1,10)=0.01	7	6
	4	F(1,10)=0.8	F(1,10)=0.1	F(1,10)=2.8	6	6
	>6	F(1,9)=0.79	F(1,9)=0.01	F(1,9)=0.001	6	5

No differences were observed among groups.

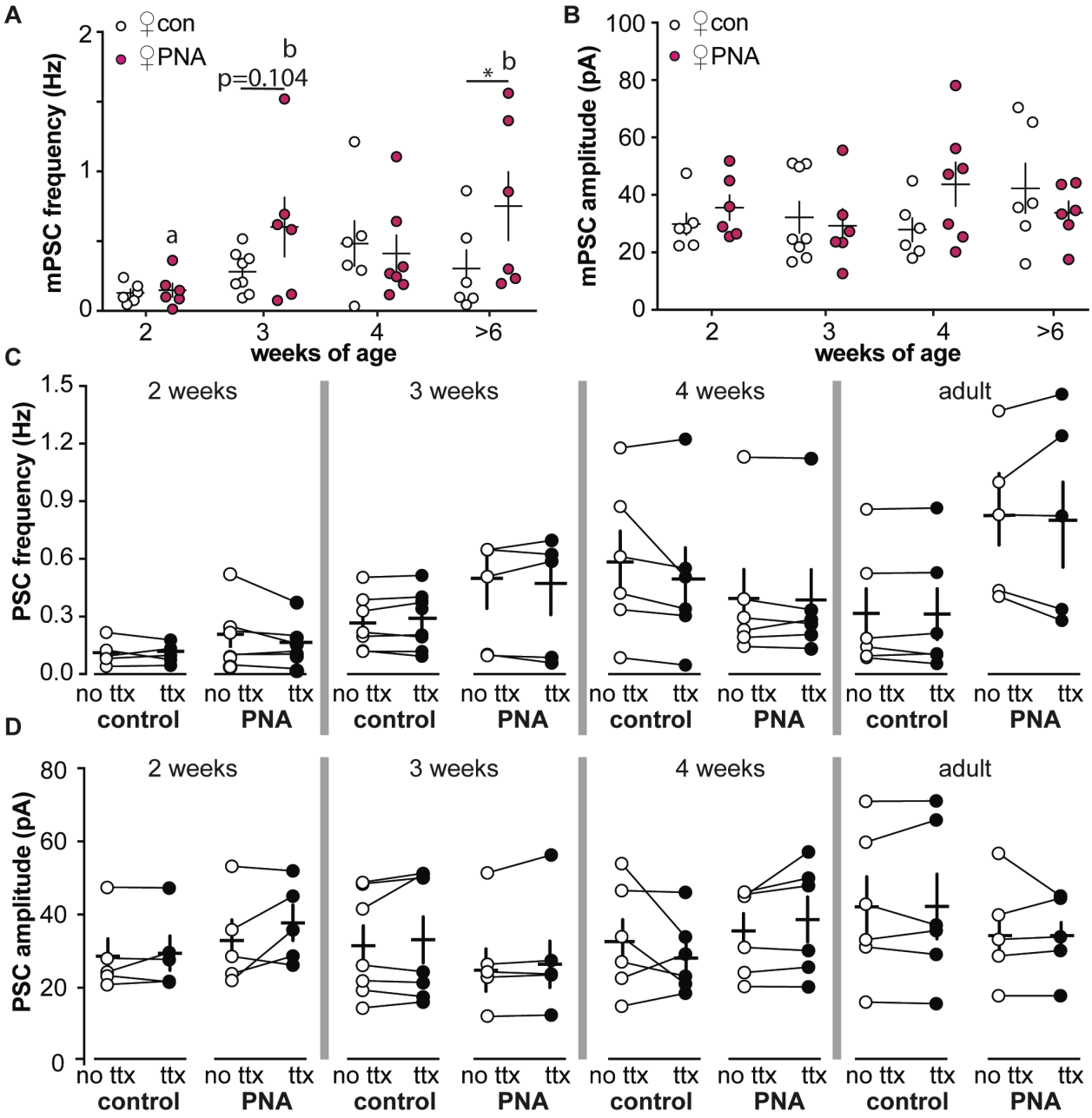

Figure 4. Activity-independent GABAergic transmission to GnRH neurons does not change throughout the prepubertal period or with PNA treatment in females. Individual values and mean ± SEM (crosses) of **A. m**PSC frequency and **B**. mPSC amplitude at 1, 2, 3, 4 weeks and adults >6 weeks. Data for control females (open circles) and PNA females (magenta circles) were compared with two-way ANOVA/Fisher's LSD. Different lower-case letters indicate differences with age within a group; * indicates $p<0.05$ between groups at each age. GABAergic PSC frequency (**C**) and amplitude (**D**) in cells under control conditions (no ttx, open circles), and in the presence of 1 μM TTX (ttx, closed circles). Data for each age were compared with two-way repeated-measures ANOVA/Fisher's LSD. Lines connect measurements made with and without TTX in the same cell.

PNA alters the functional response of GnRH neurons from prepubertal female mice to acutely applied GABA. Because our main interest was in examining potential neurobiological mechanisms underlying PCOS, further studies were confined to females. Increased GABAergic transmission frequency during development could induce compensatory changes within GnRH neurons to alter response to GABA; alternatively, altered response of GnRH neurons to GABA could lead to changes in their presynaptic network. We hypothesized that PNA affects functional response of GnRH neurons to $GABA_A$ receptor activation. To examine this, we first measured firing during an acute local application of exogenous GABA. This approach allows for rapid saturation of $GABA_A$ receptors with minimal receptor desensitization in order to study the membrane response to GABA, but does produce a longer exposure to transmitter than synaptic release provides (Figure 5A). The on-cell configuration was used to avoid disrupting the intracellular chloride concentration, upon which the response to activation of the $GABA_A$ receptor depends (Succol et al., 2012). As reported (DeFazio et al., 2002; Sullivan et al., 2003), GABA induces firing in GnRH neurons from adult control females (Figure 5B, F). In adults, PNA did not affect GnRH neuron firing response to GABA (Figure 5C, F). During development, GABA evoked firing in the majority (96%, 27 of 28) of GnRH neurons from control prepubertal mice (Figure 5D, F). In contrast, only 59% of GnRH neurons (13 of 22) from PNA mice fired in response to GABA during development (Figure 5E, F, $p<0.05$ vs con). Lack of response was concentrated at three weeks of age, with only 33% (3 of 9) of GnRH neurons firing in response to GABA ($p<0.05$ vs con), vs 83% (5 of 6) of cells from two-week old and 71% (5 of 7) of cells from four-week old PNA females (Figure 5F). The percentage of firing cells was not different between PNA and control at two ($p=0.462$) or four ($p>0.99$) weeks of age. There were no

changes in input resistance or capacitance with PNA that would contribute to the observed differences (Table 2 and 3).

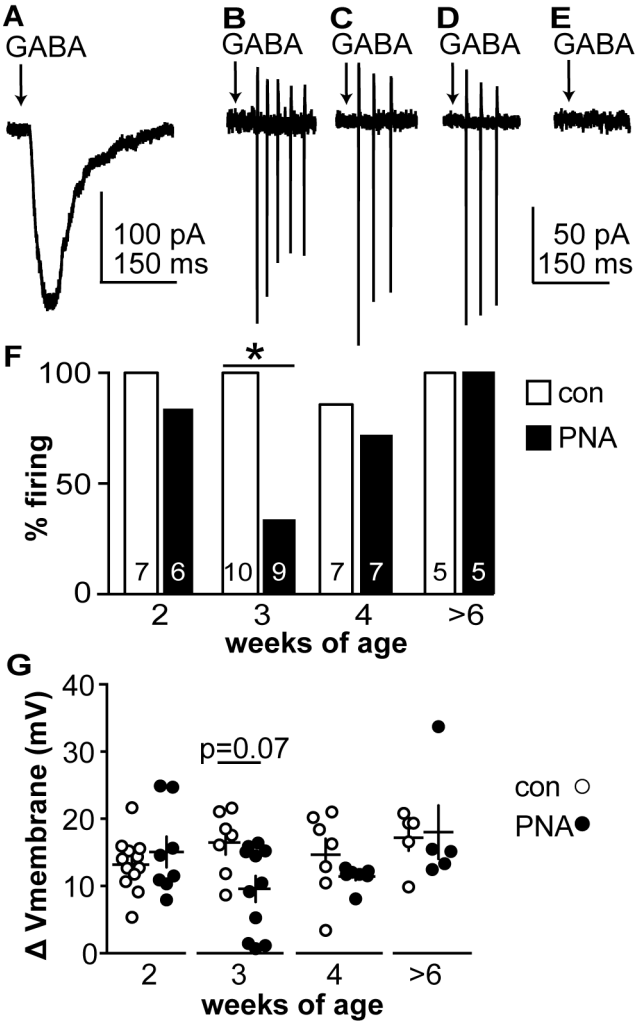

Figure 5. Response of GnRH neurons from PNA mice to acute GABA application is blunted at three weeks of age. **A**, Whole-cell voltage-clamp recording of current response to GABA in GnRH neuron from an adult control female. **B-E**, Representative on-cell recordings of firing response (vertical action currents) to GABA in cells from **B,** adult control **C**, adult PNA **D**, three-week-old control and **E**, three-week-old PNA female mice. A-E are on the same time scale. **F**, percentage of GnRH neurons that fire in response to GABA. Number of cells studied is shown within each bar (Fisher's exact test for each age group,*, $p<0.05$). **G**, individual values (circles) and mean±SEM (crosses) of the magnitude of GABA-induced membrane depolarization (two-way ANOVA/Fisher's LSD; sex/treatment $F(1, 56)=2.3$, age $F(3, 56)=2.4$, interaction $F(3, 56)=2.1$

GABA-induced membrane potential depolarization of GnRH neurons is blunted in cells from three-week old PNA mice. The altered firing response of GnRH neurons to GABA in prepubertal PNA mice is likely attributable to changes in the membrane potential response. GABA-induced current may depolarize, hyperpolarize or have no effect on membrane potential. To determine if PNA changes membrane response to GABA, we measured the membrane potential before and during response to acutely applied GABA. There was no difference in the baseline membrane potential of GnRH neurons from female mice at any age or with prenatal treatment (Table 10). GABA induced membrane depolarization in all GnRH neurons studied at all ages (Figure 5G). In GnRH neurons from PNA females, however, the magnitude of depolarization was blunted at three weeks of age compared to controls ($p<0.05$).

Table 10. Baseline membrane potential of GnRH neurons.

age (wks)	CON ♀	PNA ♀
2	-63.4±1.9 mV n=12	-65.4±2.9 mV n=8
3	-66.3±1.1 mV n=7	-61.9±1.6 mV n=11
4	-63.3±0.9 mV n=7	-63.9±1.9 mV n=7
>6	-64.7±1.8 mV n=5	-65.0±1.4 mV n=5

No differences were observed among groups; treatment $F(1, 84)=0.01$, age $F(4, 84)=0.07$, interaction $F(3, 58)=1.25$

Reversal potential for $GABA_A$-receptor-mediated current (E_{GABA}) is not altered by PNA in three-week-old females. Gramicidin perforated-patch recordings were used to measure the reversal potential for the $GABA_A$ receptor-mediated current (E_{GABA}, Figure 6). E_{GABA} was not different between control and PNA mice at three weeks of age. No difference in E_{GABA} was observed between measures made using 1 mM vs 140 mM chloride pipette solutions, indicating the

measured reversal potentials were dependent on intrinsic chloride concentration and were not contaminated by the recording.

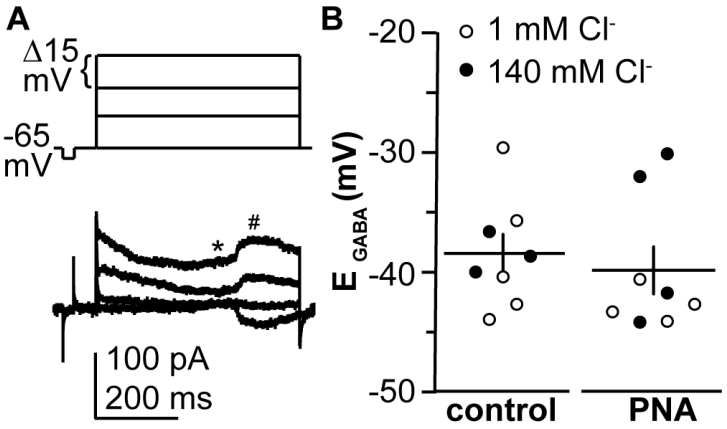

Figure 6. Reversal potential for $GABA_AR$-mediated current (E_{GABA}) is not altered by PNA in GnRH neurons from three-week-old females. **A**, Top, voltage step protocol from -65 mV to -20 mV, bottom representative gramicidin-perforated patch recording showing current response to voltage steps. * and # show where measurements were made of baseline and $GABA_AR$-mediated current, respectively. Capacitive transients at the start and end of the variable step protocol were truncated for clarity. **B**, measurements of E_{GABA} in control and PNA females. Open and closed circles show recordings using 1 mM and 140 mM chloride pipette solutions, respectively. Data analyzed using two-tailed, unpaired Student's t-test, p=0.7048.

Discussion

Neuroendocrine changes are present in most women with PCOS and similar changes have been shown to emerge during puberty in hyperandrogenemic girls (Yoo et al., 2006; Collins et al., 2014). Adult PNA mice recapitulate several features of PCOS and allow neurobiological mechanisms to be examined. GABAergic transmission to GnRH neurons is elevated in these mice in adulthood (Sullivan and Moenter, 2004). GABA levels in CSF from women with PCOS are also higher than controls (Kawwass et al., 2017). We examined effects of PNA treatment and sex on development of GABAergic transmission to GnRH neurons and their postsynaptic

response to GABA. GABAergic transmission frequency increases during prepubertal development in both sexes. In females, PNA increases transmission frequency during prepubertal development but blunts the excitatory postsynaptic response to GABA in these cells, despite not altering E_{GABA}.

In all groups, low-frequency GABAergic transmission to GnRH neurons was observed at one-week of age. Transmission frequency increased to adult levels before outward signs of puberty were observed. The present findings support and extend work showing high frequency GnRH release in brain slices from postnatal males (Glanowska et al., 2014), and a preliminary report of high frequency GnRH neuron firing activity in both sexes (Dulka and Moenter, 2016). Together these observations make a strong case that the GnRH neuronal network is active well before maturation of the downstream reproductive system. This suggests GnRH neuron activity during postnatal/pubertal development may have a role beyond reproductive output, such as sculpting formation and stabilization of nascent synapses (Katz and Shatz, 1996; Andreae and Burrone, 2014). The increase in GABAergic transmission to GnRH neurons during prepubertal development likely reflects ongoing synaptogenesis, which occurs primarily during the postnatal period in mice. For example, mature synapses in visual and somatosensory cortex increase over a similar period, from postnatal day 4 to 32 (De Felipe et al., 1997; Li et al., 2010).

In females, PNA treatment increased GABAergic transmission to GnRH neurons by three weeks of age, a difference maintained in adulthood. This increase was activity-independent, suggesting PNA alters synaptic formation and/or pruning. This observation is consistent with increased GABAergic appositions to GnRH neurons in female PNA mice by postnatal day 25 and in adults

(Silva et al., 2016). A similar increase in unidentified synaptic contacts to GnRH neurons was observed in adult PNA sheep (Kim et al., 1999). An important caveat to the present work is that some presynaptic terminals are disassociated from sites of action potential generation in brain slices. Likewise, synaptic transmission to GnRH neuron processes that are not preserved within the slice cannot be detected. There may thus be additional activity-dependent changes in transmission and/or further organizational changes within the whole brain that could not be detected.

In addition to effects on neuronal organization, PNA produces mild hyperandrogenemia in adults (Sullivan and Moenter, 2004; Witham et al., 2012; Moore et al., 2013). In control mice, gonadal biosynthesis of androgens begins around postnatal day five in females and prenatally in males (Pointis et al., 1979; Mannan and O'Shaughnessy, 1991); it is not known if this is altered by PNA. Mild elevations of androgens such as those observed in PCOS and occur in PNA mice upregulate GABA transmission frequency to GnRH neurons (Sullivan and Moenter, 2005), although high androgen doses in models of androgen abuse can reduce transmission (Penatti et al., 2010; Penatti et al., 2011). Differences in transmission observed in PNA mice could thus be attributable to a combination of altered organization and network activation by androgens. It is important to point out that prenatal androgen exposure does not merely masculinize GABAergic transmission; GABAergic transmission in PNA females is greater than control males in both three-week old and adult mice. PNA treatment of males also increases GABAergic transmission to GnRH neurons during prepubertal development, further suggesting that prenatal androgen exposure has developmental effects separate from sex differences. Unlike females, sPSC frequency was not altered by PNA in adult males. This may be attributable to differences in

androgen receptor distribution and/or levels at the time of treatment and/or later in development. Endogenous androgen levels likely also differ with sex.

In control mice of both sexes, GABA can excite GnRH neurons throughout the age range examined in the present studies (DeFazio et al., 2002). GnRH neurons from three-week-old PNA mice receive increased GABA transmission compared to controls, but a preliminary report indicates their firing activity is reduced at this age (Dulka and Moenter, 2016). This discrepancy led us to test the response of GnRH neurons to GABA in female PNA mice. Response in adult PNA mice was similar to controls. In marked contrast, cells from three-week-old PNA females fired action potentials less often and had blunted membrane depolarization in response to GABA. These observations suggest that there are postsynaptic changes in GnRH neurons from PNA mice that reduce their firing activity despite increased GABA transmission frequency at this age. This is consistent with the observation that neurons can compensate for increased excitatory drive to maintain homeostatic activity levels (Davis and Bezprozvanny, 2001).

There are several possible mechanisms by which such compensation may occur. First, PNA may alter chloride cotransporter function during development. There was no difference, however, in the reversal potential of $GABA_AR$-mediated current between three-week-old control and PNA females, suggesting any such changes have minimal perisomatic functional impact. Second, PNA may effect voltage-gated sodium channels to modify action potential threshold or increase A-type potassium currents to reduce neuronal excitability and response to synaptic inputs. Of interest with regard to the latter, testosterone increases A-type potassium current to induced arterial vasodilation (Ding and Stallone, 2001; Cairrao et al., 2008). Finally, the higher frequency

GABAergic input in PNA mice may induce short-term effects, such as shunting inhibition or partial inactivation of sodium channels, which would tend to reduce the likelihood of firing. PNA did not alter amplitude or kinetics of either mPSCs or sPSCS, suggesting that changes in postsynaptic $GABA_A$ receptors or number of these receptors activated by endogenous presynaptic GABA release do not contribute to the altered response to GABA. Regardless of the mechanisms involved in reducing GnRH neuron response to GABA in three-week-old PNA mice, these are apparently lost during development or become insufficient to reduce firing as there was no effect of PNA on response to GABA in cells from adults. Loss of these adaptive mechanisms may be an important factor in upregulating GnRH neuron output in adult PNA mice and possibly women with PCOS.

The effects of PNA are most likely dependent upon activation of androgen receptors, as the non-aromatizable androgen dihydrotestosterone (DHT) was used. In the hippocampus, androgens increase synaptic spines (Brawer et al., 1983; Hajszan et al., 2007; Hajszan et al., 2008; Hatanaka et al., 2015) and synaptic transmission during pubertal development (Pettorossi et al., 2013). Additionally, glia express androgen receptor and play key roles in synapse formation and regulation (Garcia-Segura et al., 1994; Tasker et al., 2012). Within the hypothalamus, many kisspeptin neurons in the arcuate and antereoventral periventricular nuclei are GABAergic (Cravo et al., 2011); these cells project to GnRH neurons at least in part to convey steroid feedback (Wintermantel et al., 2006). Adult males and females express androgen receptors in arcuate and AVPV kisspeptin neurons (Smith et al., 2005; Iwata et al., 2017). In males, this expression begins *in utero* in arcuate neurons but it is unknown when expression begins in females (Kumar et al., 2015). The PNA-induced changes in synaptic organization may arise from

increased connectivity with typical afferent populations such as those expressing kisspeptin and/or recruitment of additional GABAergic afferents. Although the effects of DHT are most likely through androgen receptors, we cannot rule out additional effects through activation of estrogen receptor ß (ERß) by DHT metabolites (Lund et al., 2006). ERß is expressed in GnRH neurons and its activation increases activity in these cells (Hrabovszky et al., 2000; Chu et al., 2009).

Although there were clear differences in GABAergic transmission to GnRH neurons among treatments, frequency was variable particularly in groups with higher frequencies. Both biological and technical factors may contribute to this. Differences during development may be attributable to variable timing of pubertal maturation in individual mice, and even among cells within an animal. The pattern of GABAergic transmission may change over time to help drive episodic GnRH release. Typical GnRH release intervals are long compared to the duration of recordings and cells may be in different phases when examined, contributing to a range of PSC frequencies. GABAergic populations afferent to GnRH neurons may also have different sensitivity to programming and/or activational effects of androgens.

Prepubertal development is a critical period during which the changes in GnRH neurons and their afferent network may affect the regulation of the reproductive system in adults. The increase in GABAergic transmission frequency to these cells and concomitant decrease in their responsiveness to GABA that arise before reproductive maturity in PNA females, and the subsequent loss of the postsynaptic compensation, may contribute to pathological neuroendocrine features of PNA mice and perhaps of women with PCOS.

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Chapter 3 Conclusions

Overview

The GnRH neuronal network is essential for the central regulation of fertility. Dysfunction in this network has been implicated in the pathology of polycystic ovary syndrome. The clinical finding of altered LH pulsatility in hyperandrogenemic pubertal girls gave rise to our supposition that changes in neuroendocrine networks that help regulate reproduction arise during prepubertal development. The previous chapter described the postnatal development of GABAergic inputs to GnRH neurons before reproductive maturity and demonstrated that prenatal androgenization (PNA) and sex uniquely alter GABAergic synaptic connectivity during prepubertal development. We further demonstrated that PNA alters GnRH neuron response to GABA during prepubertal development in females. These studies have broadened our understanding of the typical development of this network in both sexes and begun to explore the mechanisms by which PNA may contribute to impaired reproduction in adults. In this chapter, I will discuss further interpretations for our findings, particularly in regard to recent clinical and preclinical advances, and propose future directions.

GABAergic afferents in the arcuate nucleus may be altered by PNA

Our work shows that prenatal androgen exposure leads to increased GABAergic synaptic transmission to GnRH neurons during prepubertal development in females. These inputs were activity-independent, consistent with an increase in synaptic connectivity. An important question

to consider in interpreting the implications of these increased connections is where they originate. Broadly, increased synaptic release sites may arise from increased connectivity with existing afferents and/or through an increased number of GABAergic afferents. The latter case encompasses numerous possibilities, including expansion of existing afferent populations or recruitment of GABAergic populations that do not typically project to GnRH neurons. The most plausible sources for PNA-induced increases in GABAergic connectivity are populations that are known to send GABAergic projections to GnRH neurons, are sensitive to androgens and may relate to other features of neuroendocrine dysfunction in PNA mice. The arcuate nucleus of the hypothalamus is one such credible source.

The arcuate nucleus is considered an important mediator of hormone feedback to GnRH neurons and expresses androgen receptors in females (McAbee and DonCarlos, 1998; Yeo and Herbison, 2014). GABA is widely expressed in the arcuate nucleus and retrograde and anterograde tracing studies have demonstrated that GABAergic neurons in the arcuate robustly project to GnRH neurons (Moore et al., 2015; Zuure et al., 2016). In adult PNA compared to control females, GnRH neurons have increased appositions with GABAergic fibers that specifically originate in the arcuate (Moore et al., 2015). This finding implicates the arcuate as a likely source for at least some of the increased GABAergic transmission to GnRH neurons in prepubertal PNA mice. Interestingly, PNA does not result in any significant change in neuropeptide co-expression with GABA in the arcuate (Marshall et al., 2017). Considering this finding, increased GABAergic transmission seems less likely to arise from an increase in the number of cells within any individual subpopulation of GABAergic neurons in the arcuate and more likely to arise from increased number of synaptic release sites with existing GABAergic afferents. Additionally,

neuropeptide co-expression was found to differ between sexes and PNA did not induce a more masculinized pattern of co-expression, agreeing with our finding that PNA causes neuroendocrine changes that are distinct from sex differences (Marshall et al., 2017). While these anatomical studies appear to complement our functional studies well, the arcuate is by no means the only population of GABAergic afferents to GnRH neurons and transmission from other GABAergic afferent populations may be altered by PNA in either prepubertal and/or adult mice.

It is tempting to speculate that GABAergic afferents located in the arcuate overlap with the well characterized population of arcuate kisspeptin neurons. These neurons express AR and PR and are an important source of input to GnRH neurons (Foradori et al., 2002; Dufourny et al., 2005). Recent studies have shown that only 10-15% of arcuate kisspeptin neurons express VGAT, a marker of GABAergic neurons (Marshall et al., 2017). This was lower than an earlier report that found 50% of arcuate kisspeptin neurons express GAD-67, another marker of GABAergic neurons (Cravo et al., 2011). This difference is perhaps due to difficulty of immunodetection of kisspeptin peptide compared to using a transgenic reporter. Regardless of the rate of GABA and kisspeptin co-expression, only a minority of arcuate kisspeptin neurons actually project to GnRH neurons (Kumar et al., 2015). Although these data indicate that the population of GABAergic kisspeptin neurons afferent to GnRH neurons may not be large, it may still be physiologically important and could be altered by PNA. However, GnRH neurons in PNA compared to control ewes have been shown to have fewer appositions by fibers originating from arcuate kisspeptin neurons (Cernea et al., 2015). Furthermore, only 1% of VGAT expressing neurons in the arcuate express kisspeptin suggesting that there are likely other GABAergic afferents in the arcuate in addition to kisspeptin neurons that could be altered by PNA (Marshall et al., 2017).

Alterations in GABA signaling could contribute to neuroendocrine dysfunction seen in PCOS. Women with PCOS have elevated levels of GABA in their CSF compared to women with normal cycles in the early follicular phase, suggesting that PCOS is associated with altered GABAergic signaling (Kawwass et al., 2017). Further evidence suggesting that changes in GABA neurotransmission may play a role in the pathogenesis of neuroendocrine dysfunction in PCOS is provided by the association between epilepsy and PCOS. The anti-epileptic drug valproate increases GABA levels in the brain and may enhance GABAergic transmission through presynaptic mechanisms (Preisendorfer et al., 1987; Owens and Nemeroff, 2003). Women treated with valproate have increased risk of developing PCOS (Isojarvi et al., 1993; Sidhu et al., 2018). Although the mechanisms linking increased GABA levels and PCOS are not clear, changes in GABAergic signaling to GnRH neurons could contribute to altered hormone feedback in women with PCOS.

Both adult women with PCOS and pubertal girls with hyperandrogenemia have reduced sensitivity to progesterone negative feedback (Blank et al., 2009). Female PNA mice have similarly reduced sensitivity to progesterone feedback (Moore et al., 2015). The arcuate has been shown to be an important site for progesterone negative feedback in rodents and sheep (Goodman et al., 2011; He et al., 2017). Progesterone receptor is expressed in GABAergic arcuate neurons and GABA may help mediate progesterone feedback to GnRH neurons in mice (Leranth et al., 1992; Sullivan and Moenter, 2005). Expression of progesterone receptor is reduced in PNA mice, most notably, but not exclusively, in the arcuate nucleus (Moore et al., 2015). The mechanism by which PNA may alter GABAergic neurons in the arcuate to decrease

progesterone receptor expression and increase appositions to GnRH neurons is not clear. Together, increased GABAergic transmission and reduced sensitivity to suppression by progesterone feedback are potential drivers of increased GnRH neuron activity women and girls with PCOS.

Projections from neurons in the arcuate nucleus have been shown to develop postnatally in mice (Bouret et al., 2004). Tracing of arcuate neuron axons showed that innervation of the medial preoptic area does not reach mature levels until two weeks of age (Bouret et al., 2004). Formation of functional synapses likely follows the first appearance of axonal projections. The timing of the development of efferent projections from the arcuate is interesting firstly, because it mirrors the timing of development of GABAergic transmission to GnRH neurons in control females and secondly because if arcuate neuron projections to GnRH neurons are altered by PNA it would help explain our observation that PNA does not increase the frequency of activity-independent GABAergic transmission to GnRH neurons until three-weeks of age. Although the density of labeled axonal fibers did not change between two weeks of age and adulthood, there could still be changes in synaptic connectivity due to increased formation or pruning of the number of synaptic release sites (Chan et al., 2011). Although the study by Bouret et al. did not specifically examine GABAergic arcuate neurons, it shows that development of synaptic innervation from the arcuate nucleus likely occurs at least to some degree during prepubertal development. In order to determine if the altered GABAergic transmission that emerges during this time is dependent on concomitantly elevated androgen levels we could treat PNA and control mice with a placebo or an androgen receptor antagonist such as flutamide and determine if this ameliorates changes with PNA.

GABAergic transmission is not the only source of excitatory input to GnRH neurons. Glutamatergic neurons also provide fast-synaptic transmission to GnRH neurons (Ottem et al., 2002; Christian et al., 2009) and may be altered by PNA either in prepubertal and/or adult mice. GnRH neurons from adult PNA mice do not have an increase in the density or number of dendritic spines with close appositions with glutamatergic fibers (Moore et al., 2015), however PNA may program differences in activity-dependent synaptic transmission during development and/or adulthood. The timing of development of glutamatergic transmission to GnRH neurons is not known nor is it known if PNA changes glutamatergic synaptic connectivity during prepubertal development. A decrease in glutamatergic EPSCs, alone or in combination with GnRH neuron intrinsic changes, could in part contribute to decreased GnRH neuron firing at three-weeks of age (Dulka and Moenter, 2016). Future studies could examine the frequency and amplitude of glutamatergic currents in control and PNA females at three-weeks of age and in adults to determine if changes in the glutamatergic afferent neuronal network may play a role in altered GnRH neuron activity in PNA mice. Additional aspects of the GnRH afferent neuronal network that could be altered by PNA include non-GABAergic, non-glutamatergic kisspeptin neurons in the arcuate and/or AVPV nuclei. For example, anatomical tracing studies could be used to examine if PNA changes the density of kisspeptin projections near GnRH neurons or functional studies could be used to measure if PNA changes kisspeptin release in the area of GnRH neurons or their projections to the median eminence.

The effects of androgens may be mediated by different populations such as glutamatergic, GABAergic and/or kisspeptidergic neurons that project directly and indirectly to GnRH neurons.

To better understand how each of these populations may contribute to the effects of PNA, future studies could selectively remove androgen receptor from each of these cell types in control and PNA mice and measure if AR receptor knock out in a given population is sufficient to blunt or diminish the changes induced by PNA. This would give more targeted information than global AR knock out or systemic treatment with an androgen receptor antagonist such as flutamide. Two approaches could be used to delete androgen receptor from specific populations of interest. First, floxed AR transgenic mice could be crossed with cell type specific CRE transgenic mice, such as VGAT CRE mice. This approach would allow for a AR to be knocked out in a high percentage of GABAergic cells but would likely include cells that are not part of the GnRH afferent network. In the case of GABA, AR would primarily be knocked out centrally rather than in the gonads and elsewhere. Kisspeptin, however, has been shown to be expressed outside of the brain and these cells may also theoretically express AR. Furthermore, this knock out would occur before birth rather than specifically examining androgen receptor-dependent effects during prepubertal development.

To refine our understanding of the effects of AR signaling in different aspects of the GnRH neuronal network a second, more spatially and temporally targeted approach could be used. Floxed AR transgenic mice could be injected with an AAV-CRE virus to specific nuclei of interest and at different times during postnatal development, potentially as early as three weeks of age. Notably, this approach would not be selective for specific cell types but would target all AR-expressing cells in a nucleus. This approach would have several advantages, particularly as a followup to the first approach, in that it would allow for knock out of AR in specific nuclei known to project to GnRH neurons, such as the arcuate. This approach could also be used to

target AR in other nuclei that express GABA such as those that contain populations of AGRP or POMC neurons. It is likely that AR expression would be knocked down but not deleted entirely which is desirable since it would be more physiologically comparable to the relative difference in androgen exposure in control and PNA mice. Reproductive outcomes to be compared could include vaginal opening, first estrous, fertility and estrous cycle characteristics.

Interesting future studies could also expand our understanding of the development of GABAergic transmission to GnRH neurons. Our studies were done in coronal brain slices in which afferent cell bodies that are not in the slice are separated from their synaptic terminals. Alternative slice orientations could be used to preserve intact connections with afferents in the arcuate nucleus or elsewhere. Another limitation to this work is that it is difficult to maintain a stable whole-cell recording configuration for long periods of time. Recordings of GnRH neuron firing rates last for minutes to hours while recordings of PSCs rarely last more than 30 minutes and are sometimes less than 10 minutes (following stabilization). Longer recordings could reveal if there are changes in transmission frequency or amplitude that occur over time and give us a more complete picture of the function of the afferent network to GnRH neurons. Additionally, the pattern of PSCs, not only the frequency itself, may be important to determining the effect of inputs on cell firing and/or GnRH release.

Potential mechanisms of decreased GnRH neuron response to GABA in prepubertal PNA mice

A recent paper from our lab demonstrated that GnRH neuron firing rates are decreased in PNA mice at 3-wks of age, despite increase in GABAergic transmission (Dulka and Moenter, 2016).

This contrasts with PNA adults in which increased GABAergic transmission frequency is associated with increased GnRH neuron firing rate (Roland and Moenter, 2011). We showed that GnRH neurons from three-week old, but not adult, PNA mice have reduced membrane potential depolarization in response to GABA and are less likely to fire in response to GABA. The blunted membrane potential response to GABA in GnRH neurons from prepubertal PNA mice may indicate that PNA induces changes in the responsiveness of GnRH neurons to $GABA_AR$ activation. GABA receptor subtypes may change during prepubertal development (Sim et al., 2000) and PNA could alter the expression of different GABA receptor subtypes or alter their binding affinity, duration of ion channel opening or the open probability of the ion channel. In addition to $GABA_AR$ on the postsynaptic membrane of GnRH neurons, synaptic glial cells are important for rapidly clearing the high concentrations of GABA in the synaptic cleft (Gadea and Lopez-Colome, 2001; Tasker et al., 2012). Glial cells express AR and could theoretically be altered to clear GABA more or less efficiently (Garcia-Ovejero et al., 2002). However, such changes were not indicated by our data as we did not observe any changes in PSC amplitude or decay time. We hypothesized that PNA could alter chloride homeostasis during development as a mechanism to explain the change in membrane depolarization in response to GABA. However, we did not observe any differences in the equilibrium potential of $GABA_AR$-associated current between control and PNA prepubertal mice. Furthermore, the $GABA_AR$ equilibrium potentials measured in prepubertal mice were no different from adults in either control or PNA groups and no different from previously published adult values (unpublished data).

The change in firing response to GABA may be explained by other changes in the intrinsic properties of GnRH neurons. Intrinsic properties refer to the expression and activity of ion

channels and transporters that help determine the basal membrane potential and membrane potential changes of a neuron in response to current. In prepubertal PNA mice, changes in intrinsic properties could underlie the altered response to GABA and/or contribute to increased synaptic connectivity through altered synaptic refinement (i.e., stabilization and/or pruning). Regardless of the mechanism, intrinsic changes may contribute to altered function of GnRH neurons and their network in prepubertal PNA mice. Intrinsic properties that could be altered by PNA include excitability and action potential properties. Excitability is measured as the minimal current necessary to induce firing and the number of spikes generated by depolarizing current steps. Action potential properties include the latency to fire between the start of a current injection to the first spike, the spike amplitude and full width at half maximum (FWHM), spike rate of rise, firing threshold and amplitude of the after hyperpolarization (AHP). Decreased excitability, increased latency to fire and depolarization of the firing threshold could all contribute to the decreased firing response to GABA in GnRH neurons in prepubertal PNA mice. If such developmental changes in excitability and action potential properties exist in prepubertal PNA mice such differences either don't persist into adulthood or are overcome by increased excitatory inputs to result in increased GnRH neuron firing. GnRH neuron intrinsic properties have not been studied during pubertal development or in PNA mice at any stage of development.

Effects of PNA on GnRH neuron intrinsic properties could be mediated by subthreshold currents. A-type potassium currents as well as calcium currents are important for determining excitability and action potential properties in GnRH neurons and are modulated by steroid hormone feedback in adults (DeFazio and Moenter, 2002). A-type potassium current may be activated by small membrane depolarizations in response to GABAergic transmission. Increased A-type potassium

current by PNA could prevent further depolarization to threshold and thus limit firing in response to GABA.

Effects of testosterone treatment in female-to-male transgender adolescents

Gender dysphoria is a condition in which the gender assigned at birth is incongruent with the individual's self-perception of their gender (Wiepjes et al., 2018). Testosterone replacement is an important aspect of gender-affirming treatment for transgender males (female-to-male, FTM). Testosterone is administered in doses sufficient to achieve serum levels within the normal physiologic range for males (Hembree et al., 2017). This treatment is continued long-term to induce and maintain virilization of secondary sex characteristics (den Heijer et al., 2017). Although the availability and access to health care professionals who are appropriately trained and willing to care for transsexual individuals has increased over the last several decades, the impact of testosterone treatment on the hypothalamic-pituitary-ovarian axis has not been well studied in this population.

Multiple studies in female-to-male transsexuals have shown that testosterone treatment results in morphological changes in the ovaries similar to changes seen in PCOS (Futterweit and Deligdisch, 1986; Spinder et al., 1989; Grynberg et al., 2010). The majority of these patients had complete cessation of regular menses. However, these studies were done on adults with a wide range in both age and duration of treatment. Treatment of adult (6-13 years of age) rhesus monkeys for only 10 days resulted in enhanced ovarian follicle growth, suggesting that ovarian changes may begin relatively early after treatment initiation (Vendola et al., 1998). The effects of testosterone treatment may depend on when therapy is initiated relative to the pubertal transition.

As discussed previously, exposure to androgens, particularly during development, may play a role in the pathogenesis of PCOS (Burt Solorzano et al., 2010). Interestingly, three-week old female rats treated with DHEAS for two weeks developed ovarian cysts (Anderson et al., 1992; Honnma et al., 2006). Currently the standard of care for treatment of FTM individuals in early adolescence is to administer GnRH agonists i.e., 'puberty blockers' at the initiation of puberty (Tanner stage 2) and to begin testosterone treatment at around age 16 while they are still biologically in the early stages of puberty (Hembree et al., 2017). While some features of clinical hyperandrogenemia are desirable in these patients, such as male-pattern hair growth, other aspects of PCOS, including the associated metabolic syndrome, would be detrimental. Even if testosterone therapy does not induce PCOS in FTM transsexuals, it could exacerbate symptoms in those with underlying PCOS or susceptibility to PCOS and potentially worsen metabolic features. Additionally, medical professionals have historically assumed that these individuals had no desire for fertility, however there is emerging understanding that many of these individuals do desire to have their own children. The possible association between adolescent androgen exposure and PCOS should be further explored so that patients can be appropriately counseled on the risks and benefits of initiating testosterone therapy.

Final conclusions

Exposure to androgens during development has been shown to result in an adult phenotype that recapitulates features of PCOS and our work adds to our understanding that this effect may be in part mediated through androgen effects on prepubertal development of neuronal networks afferent to GnRH neurons. Development of a PCOS-like phenotype in adult PNA mice is dependent on neuronal expression of androgen receptor suggesting that early androgen exposure

directly impacts developing neuronal networks (Caldwell et al., 2017). In young pubertal female mice, exposure to an aromatase inhibitor, Letrozole, is sufficient to recapitulate neuroendocrine features of PCOS including altered gonadotropin secretion and reduced progesterone receptor expression (Kauffman et al., 2015).

Childhood obesity and its associated hyperandrogenemia may be a predisposing factor for developing PCOS and/or increase the severity of PCOS in adolescence (Anderson et al., 2014). Overweight peripubertal girls have increased androgens from both adrenal and ovarian sources (Burt Solorzano et al., 2017). Prepubertal androgen exposure, regardless of the source, may alter gonadotropin secretion and promote further excess androgen production. Obesity in peripubertal girls may therefore create a vicious cycle of neuroendocrine dysfunction and hyperandrogenemia. Impaired reproductive function may therefore be yet another comorbidity of the childhood obesity epidemic. Increasing our understanding of the role of pubertal development in the pathogenesis of PCOS will create greater opportunities for earlier intervention and management of PCOS symptoms.

Although precursors to PCOS have been established to emerge before pubertal maturation, the difficulty in diagnosing PCOS in early pubertal girls results in treatment being delayed until later in adolescence when the diagnosis can be well established. Hyperandrogenemia may be the earliest clinically useful indicator of the risk of developing PCOS in adolescents. Postmenarchal girls have irregular cycles with frequent anovulatory cycles for several years following menarche (Knudsen et al., 2010; Shayya and Chang, 2010). Polycystic ovaries are not recommended as a diagnostic feature for PCOS in adolescents as advances in ultrasound technology have made

possible the realization that PCO is a common finding in young women (Kristensen et al., 2010). Furthermore, transabdominal ultrasound is less sensitive in obese patients and transvaginal ultrasound is not appropriate for the evaluation of young pubertal girls, particularly those who are not sexually active. Interventions aimed at identifying and decreasing excess androgen levels in obese prepubertal girls may be a strategy for PCOS prevention.

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