

Prepubertal Development of GABAergic Transmission to Gonadotropin-Releasing Hormone (GnRH) Neurons and Postsynaptic Response Are Altered by Prenatal Androgenization

Tova Berg,¹ Marina A. Silveira,⁴ and Suzanne M. Moenter^{1,2,3}

Departments of ¹Molecular and Integrative Physiology, ²Internal Medicine, ³Obstetrics and Gynecology, University of Michigan, Ann Arbor, Michigan 48109, and ⁴Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, CEP 05508-900, São Paulo, Brazil

Gonadotropin-releasing hormone (GnRH) neurons regulate reproduction through pulsatile GnRH release. Women with polycystic ovary syndrome (PCOS) have persistently elevated luteinizing hormone release frequency, reflecting GnRH release; this exacerbates hyperandrogenemia and disrupted reproductive cycles that are characteristic of this disorder. Clinical evidence suggests that 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 GABAergic transmission, which is excitatory. When changes emerge during development is unknown. To study the typical postnatal development of GABAergic transmission and the 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 1 week of age in females and males and increased in frequency, reaching adult levels at 3 and 4 weeks, respectively. GABAergic PSC frequency was elevated in 3-week-old PNA versus control females. PSC frequency in both controls and PNA mice was activity independent, suggesting that PNA induces changes in synapse organization. PNA also alters the functional response of GnRH neurons to GABA. GABA induced firing in fewer neurons from 3-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.

Key words: GABA; GnRH; gramicidin; PCOS; PNA

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 midpubertal girls suggests that 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 gonadotropin-releasing hormone (GnRH) neurons is important for reproduction and is increased in adult PNA mice. The typical development of this network and when changes with PNA and sex arise relative to puberty are unknown. 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.

Introduction

Gonadotropin-releasing hormone (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 to drive differential synthesis and the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary (Wildt et

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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 both altered in women with polycystic ovary syndrome (PCOS). PCOS is a leading cause of infertility, conservatively affecting 8% of women of reproductive age (Goodarzi et al., 2011). Women with PCOS have persistently elevated GnRH/LH release frequencies 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 the elevated ovarian androgen production and irregular menstrual cycles that are 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 seen in midpubertal 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 frequencies, 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 levels even in adulthood and thus exhibit an excitatory response to GABA action via GABA_A receptors (GABA_ARs; 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 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 14:10 light/dark cycle with lights on at 4:00 A.M. Eastern Standard Time. To generate PNA mice, a GnRH-GFP mouse and a CD1 female mouse were paired for 1–3 weeks and then a stud male was introduced. Males were removed after pregnancy was established. PNA mice were generated by injecting pregnant GnRH-GFP dams with dihydrotestosterone (DHT; 225 μ g, s.c., 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; as 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 the survival times of PNA pups. Litter sizes were adjusted to <15 pups by culling CD1 pups to normalize nutrition. Pups not used for postnatal recordings were weaned at 3 weeks of age. Androgenization of PNA mice was confirmed in surviving female littermates by younger age of vaginal opening (con mice, 32.9 ± 0.6 d old, $n = 20$ vs PNA mice, 28.7 ± 0.4 d old, $n = 21$; $p < 0.05$), disrupted estrous cycles (time spent in estrus: con mice, 23%, $n = 12$; vs PNA mice, 8%, $n = 12$; $p < 0.05$) and greater anal–genital distance (con mice, 5.5 ± 0.2 , $n = 12$; vs PNA mice, 6.6 ± 0.3 , $n = 12$; $p < 0.05$). Female and male mice at postnatal ages 1, 2, 3, and 4 weeks ± 1 d and adults (42–120 d old) were used for recording experiments. Experiments on female mice that had attained puberty were performed 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 min 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 the following (in mM): 250 sucrose, 3.5 KCl, 26 NaHCO₃, 10 D-glucose, 1.25 Na₂HPO₄, 1.2 MgSO₄, and 3.8 MgCl₂. Coronal slices (300 μ m) were made using a Leica BiosystemsVT12000S Vibratome. Slices were incubated at room temperature for 30 min in a 50:50 solution of sucrose saline and ACSF containing the following (in mM): 135 NaCl, 3.5 KCl, 26 NaHCO₃, 10 D-glucose, 1.25 Na₂HPO₄, 1.2 MgSO₄, and 2.5 CaCl₂, 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 h 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 $30 \pm 1^\circ\text{C}$. ACSF contained 20 μ M CNQX and 20 μ M 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 (outer diameter, 1.65 mm; inner diameter, 1.1 mm; World Precision Instruments) using a P-97 puller (Sutter Instruments). Pipettes were filled with a solution containing (in mM): 140 KCl, 10 HEPES, 5 EGTA, 0.1 CaCl₂, 4 MgATP, and 0.4 NaGTP, pH 7.2, at 310 mOsm/L. Pipettes with resistances of 2–4 M when filled this pipette solution were

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Correspondence should be addressed to Suzanne M. Moenter, Department of Molecular and Integrative Physiology, University of Michigan, 7725 Med Science II, 1137 East Catherine Street, Ann Arbor, MI 48109-5622. E-mail: smoenter@umich.edu.

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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) running on a Macintosh G4 computer (Apple). Membrane currents were filtered at 6.5 kHz and digitized at 10 kHz. Cell location was noted and mapped to an atlas to determine whether cell location-based differences emerged (Paxinos and Franklin, 2001). No differences in response attributable to rostral–caudal and medial–lateral distributions were evident. No more than three cells were used per animal. The variability between GnRH neurons recorded from a single mouse was similar to the variability of GnRH neurons among mice.

GABAergic PSC recordings. Whole-cell voltage-clamp recordings were made following the formation of a high-resistance seal (≥ 1.3 G Ω) between the pipette tip and cell membrane. GABAergic spontaneous PSCs (sPSCs) were recorded at -55 mV (number of cells per group are shown in Table 1). In some cells, activity-independent miniature PSCs (mPSCs) were recorded in the presence of $1 \mu\text{M}$ tetrodotoxin (TTX, Tocris) following the measurement of sPSCs. To monitor cell health and recording quality, input resistance (>500 M Ω), series resistance (<20 M Ω uncompensated, $<20\%$ change during the analyzed recording period), holding current (-60 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 (Table 2, 3). Capacitance increased with age but was not altered by sex or PNA treatment. These changes would not be expected to affect the data reported, and no other changes in passive properties were observed.

GABA application. Local pressure application of GABA was accomplished as described previously (DeFazio et al., 2014). Pipettes were filled with $100 \mu\text{M}$ GABA in a HEPES-buffered saline solution containing the following (in mM): 150 NaCl, 10 HEPES, 10 D-glucose, 3.5 KCl, 2.5 CaCl₂, and 1.3 MgCl₂, pH 7.2 with NaOH. This pipette was placed $\sim 15 \mu\text{m}$ from the soma, and a pulse of 5–10 psi was delivered using a PV820 picospritzer (World Precision Instruments). To determine whether 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 previously (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 ms. 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 the 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 the 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 the study by 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°C. A working concentration (50–100 $\mu\text{g}/\text{ml}$) was made fresh for each cell in pipette solution and sonicated for 10–20 s immediately before use. Patch pipettes (2–4.5 M Ω) were backfilled with 1.5–2.5 μl of gramicidin-free pipette solution followed by gramicidin solution. Two pipette solutions were used, one with high chloride levels and one with low chloride levels; values obtained with these two solutions will be similar if inadvertent patch rupture has not occurred. The low-chloride (1 mM) pipette solution consisted of the follow-

Table 1. Cells/group for sPSC recordings

Age (weeks)	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

Group	Age (weeks)	Input resistance	Series resistance	Membrane capacitance	Holding current
♀ 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

Values are the mean ± SEM. Different lower case letters indicate differences with age within a group. Significant differences are 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	$F_{(1,73)} = 1.58$	$F_{(4,73)} = 1.92$	$F_{(4,73)} = 0.53$

* $p < 0.05$; ** $p < 0.01$.

ing (in mM): 139 K gluconate, 1 KCl, 10 HEPES, 5 EGTA, 0.1 CaCl₂, 4 MgATP, and 0.4 NaGTP, pH 7.2, at 310 mOsm/L. For the high-chloride (140 mM) pipette solution, K gluconate was replaced with KCl. The membrane potential for recordings with 1 or 140 mM chloride were corrected for liquid junction potentials of 16.5 and 4.9 mV, respectively.

After obtaining a minimum resistance seal of 1 G Ω , 5 mV hyperpolarizing steps from a holding potential of -65 mV were made in voltage clamp to monitor membrane perforation. Recordings were initiated

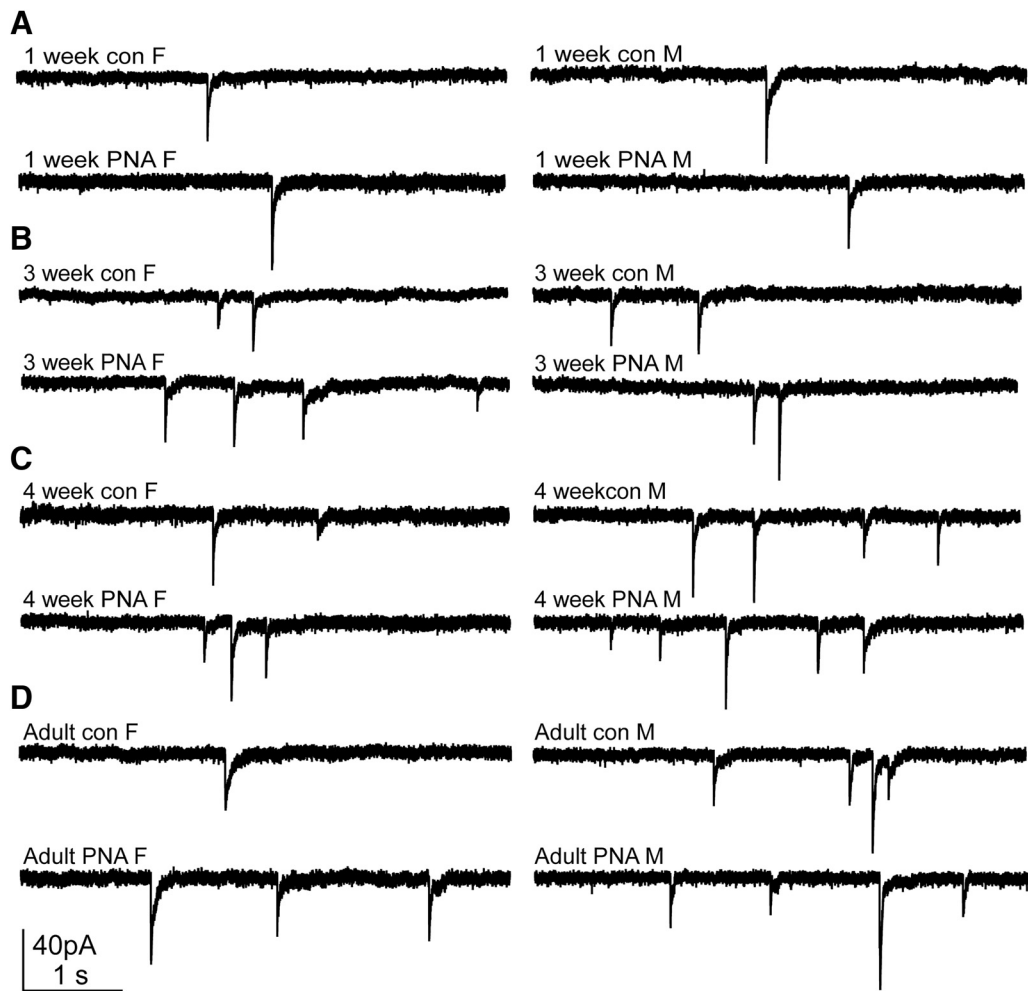


Figure 1. Representative recordings of GABAergic PSCs in GnRH neurons in female (F; left) and male (M; right) control (top) and PNA (bottom) mice at ages 1 week (A); 3 weeks (B); 4 weeks (C); and adults >6 weeks (D).

when the series resistance fell to $<50 \text{ M}\Omega$. Voltage steps (-80 to $+5 \text{ mV}$, 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 custom software written in IGOR Pro (Wavemetrics) and was verified by eye for each recording. Only reversals that were stable ($<5 \text{ mV}$ variation) for at least two complete measurements were accepted. The rupture of perforated-patch recordings to whole-cell mode was detected by an abrupt decrease in access resistance, an increase in membrane capacitance, a broadening on capacitive transient shape, and a rapid change in the measured reversal potential (hyperpolarized with a 1 mM chloride pipette solution, depolarized with a 140 mM chloride pipette solution). Any recordings in which any of these changes were detected were discarded.

Analysis. PSC data were analyzed using custom software (Sullivan et al., 2003; DeFazio et al., 2014) written in IgorPro. 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 \pm 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 as the mean \pm SEM. Data were examined for normal distribution using the Shapiro–Wilk test. Statistical analyses were performed (Prism, GraphPad Software) 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 (LSD) *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. Representative recordings are shown in Figure 1, and group comparisons are shown in Figure 2 and Table 4. Low-frequency GABAergic transmission was observed at 1 week of age in all cells studied. In cells from control females, GABAergic transmission frequency increased between 2 and 3 weeks of age ($p < 0.05$; Fig. 2A). In cells from control males, an increase was

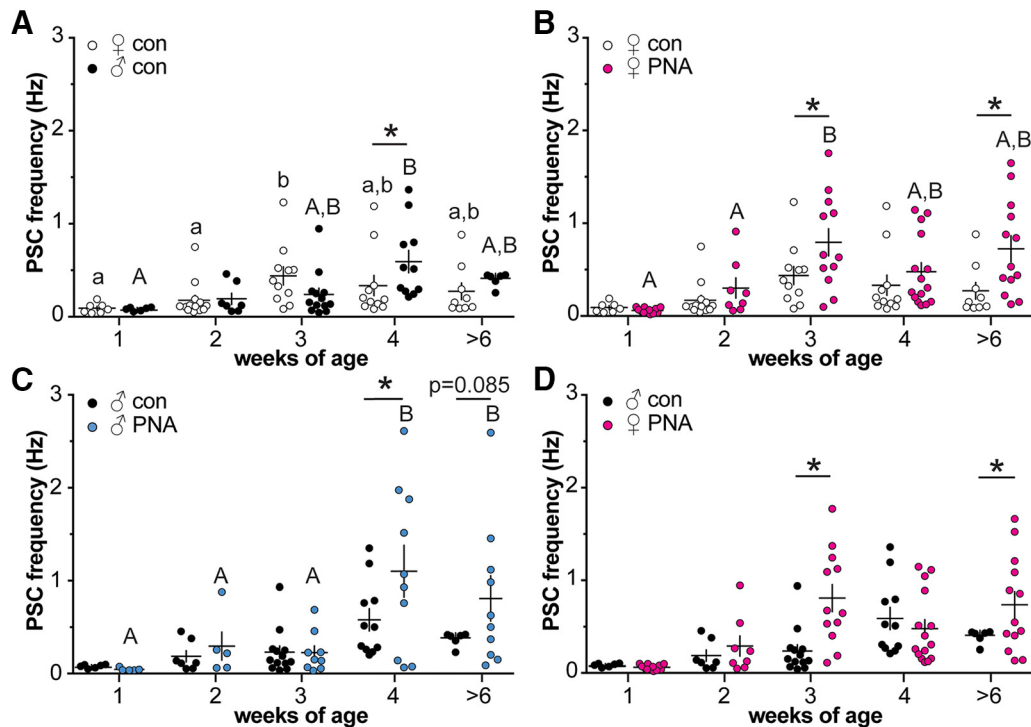


Figure 2. Spontaneous GABAergic transmission frequency to GnRH neurons changes throughout the prepubertal period in both sexes and is altered by PNA. **A–D**, Individual values and mean \pm SEM of GABAergic PSC frequency at 1, 2, 3, and 4 weeks and adults at >6 weeks. **A**, Control females (open circles)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

Table 4. Two-way ANOVA parameters for comparison of sPSC and mPSC frequency and amplitude among groups

Parameter (figure displaying data)	Sex/treatment	Age	Interaction
sPSC frequency (Fig. 2A)	$F_{(1,84)} = 1.72$	$F_{(4,84)} = 5.64^{***}$	$F_{(4,84)} = 2.20^*$
sPSC amplitude (Fig. 3A)	$F_{(1,84)} = 0.17$	$F_{(4,84)} = 1.76$	$F_{(4,84)} = 1.30$
sPSC frequency (Fig. 2B)	$F_{(1,102)} = 9.45^{**}$	$F_{(4,102)} = 8.44^{****}$	$F_{(4,102)} = 1.57$
sPSC amplitude (Fig. 3B)	$F_{(1,102)} = 0.01$	$F_{(4,102)} = 0.5$	$F_{(4,102)} = 0.7$
sPSC frequency (Fig. 2C)	$F_{(1,73)} = 3.63^*$	$F_{(4,73)} = 7.77^{****}$	$F_{(4,73)} = 1.24$
sPSC amplitude (Fig. 3C)	$F_{(1,73)} = 0.07$	$F_{(4,73)} = 3.03^*$	$F_{(4,73)} = 0.88$
sPSC frequency (Fig. 2D)	$F_{(1,94)} = 6.37^*$	$F_{(4,94)} = 7.14^{****}$	$F_{(4,94)} = 3.73^{**}$
sPSC amplitude (Fig. 3D)	$F_{(1,94)} = 0.20$	$F_{(4,94)} = 0.82$	$F_{(4,94)} = 1.80$
mPSC frequency (Fig. 5A)	$F_{(1,43)} = 3.63^*$	$F_{(3,43)} = 2.78^*$	$F_{(3,43)} = 1.47$
mPSC amplitude (Fig. 5B)	$F_{(1,43)} = 0.37$	$F_{(3,43)} = 0.61$	$F_{(3,43)} = 1.62$

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

not observed until 4 weeks of age ($p < 0.05$). PSC frequency was higher in males than females at 4 weeks of age ($p < 0.05$).

PNA alters prepubertal development of GABAergic transmission to GnRH neurons in females and males

In cells from PNA females, GABAergic transmission frequency increased between 2 and 3 weeks of age as in controls, but the increase was more robust in PNA females (Fig. 2B). As a result, PSC frequency was elevated in cells from PNA compared with control females at 3 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 4 weeks of age in cells from PNA compared with control males ($p < 0.05$), but not in adulthood ($p = 0.085$; Fig. 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

(Fig. 2D). Cells from PNA females had higher GABAergic PSC frequency than cells from control males at 3 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 (Fig. 3, Table 4). There was no overall trend with age, sex, or treatment in sPSC kinetics (Tables 5, 6). The FWHM was smaller at 4 weeks in control females and thus was elevated in controls at 2 weeks of age and in adults.

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 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 with controls; in 3-week-old females, mPSC frequency did not meet the criteria for statistical significance ($p = 0.104$; Fig. 4A, Table 4). Neither the amplitude nor the kinetics of mPSCs differed with age or PNA treatment (Fig. 4B, Tables 4, 7, 8). Similarly, comparison of sPSC and mPSC frequency and amplitude in the same cell revealed no difference with age or PNA treatment (Fig. 4C,D, Table 9).

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 con-

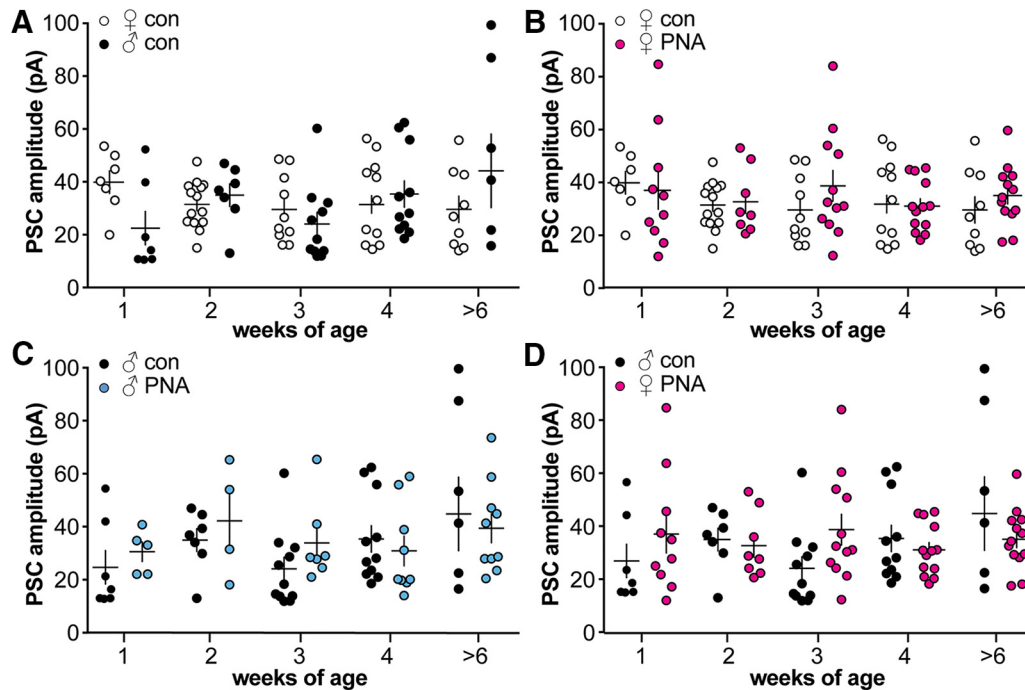


Figure 3. The amplitude of GABAergic sPSCs is not altered by PNA or sex. **A–D**, Individual values and mean \pm SEM (crosses) of GABAergic PSC amplitude at 1, 2, 3, and 4 weeks and adults at >6 weeks. No differences were seen when analyzed using a two-way ANOVA/Fisher’s LSD. **A**, Control females (open 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. Kinetic parameters of GABA sPSCs in GnRH neurons

Group	Age (weeks)	Rise time (ms)	Decay (ms)	FWHM (ms)
♀ Con	1	0.37 \pm 0.06	17.2 \pm 2.2	5.99 \pm 0.75
	2	0.38 \pm 0.06	18.8 \pm 1.0	6.72 \pm 0.53 ^a
	3	0.52 \pm 0.08	17.0 \pm 1.4	5.42 \pm 0.32
	4	0.49 \pm 0.09	19.2 \pm 1.5	4.17 \pm 0.65 ^b
	>6	0.43 \pm 0.11	19.4 \pm 1.5	6.41 \pm 0.64 ^a
♀ PNA	1	0.52 \pm 0.17	18.8 \pm 3.5	5.94 \pm 0.57
	2	0.50 \pm 0.10	23.1 \pm 2.4	6.69 \pm 0.57
	3	0.32 \pm 0.07	18.4 \pm 1.7	4.99 \pm 0.58
	4	0.49 \pm 0.05	17.4 \pm 1.0	5.92 \pm 0.38
	>6	0.42 \pm 0.07	20.9 \pm 1.0	6.02 \pm 0.82
♂ Con	1	0.62 \pm 0.14	22.6 \pm 3.2	7.57 \pm 0.13
	2	0.40 \pm 0.08	20.4 \pm 3.0	6.69 \pm 0.57
	3	0.31 \pm 0.10	18.2 \pm 2.8	7.73 \pm 1.06
	4	0.33 \pm 0.09	20.6 \pm 2.0	5.92 \pm 0.38
	>6	0.22 \pm 0.09	20.7 \pm 1.5	6.02 \pm 0.82
♂ PNA	1	0.35 \pm 0.08	23.3 \pm 2.8	6.39 \pm 0.61
	2	0.29 \pm 0.09	25.2 \pm 0.9	8.30 \pm 0.59
	3	0.57 \pm 0.05	18.2 \pm 2.7	5.38 \pm 0.52
	4	0.48 \pm 0.08	20.2 \pm 1.1	6.76 \pm 0.51
	>6	0.36 \pm 0.05	21.9 \pm 2.2	5.99 \pm 0.46

Values are the mean \pm SEM. 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.

finned to females. Increased GABAergic transmission frequency during development could induce compensatory changes within GnRH neurons to alter the response to GABA; alternatively, the altered response of GnRH neurons to GABA could lead to changes in their presynaptic network. We hypothesized that PNA affects the 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 to study the membrane response to

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	$F_{(1,73)} = 0.41$	$F_{(4,73)} = 2.21$	$F_{(4,73)} = 0.60$
	FWHM	$F_{(1,73)} = 0.30$	$F_{(4,73)} = 0.40$	$F_{(4,73)} = 0.40$

* $p < 0.05$.

GABA, but does produce a longer exposure to transmitter than synaptic release provides (Fig. 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 previously (DeFazio et al., 2002; Sullivan et al., 2003), GABA induces firing in GnRH neurons from adult control females (Fig. 5B,F). In adults, PNA did not affect the GnRH neuron firing response to GABA (Fig. 5C,F). During development, GABA evoked firing in the majority of GnRH neurons (96%, 27 of 28 neurons) from control prepubertal mice (Fig. 5D,F). In contrast, only 59% of GnRH neurons (13 of 22 neurons) from PNA mice fired in response to GABA during development (Fig. 5E,F; $p < 0.05$ vs cons). Lack of response was concentrated at 3 weeks of age, with only 33% of GnRH neurons (3 of

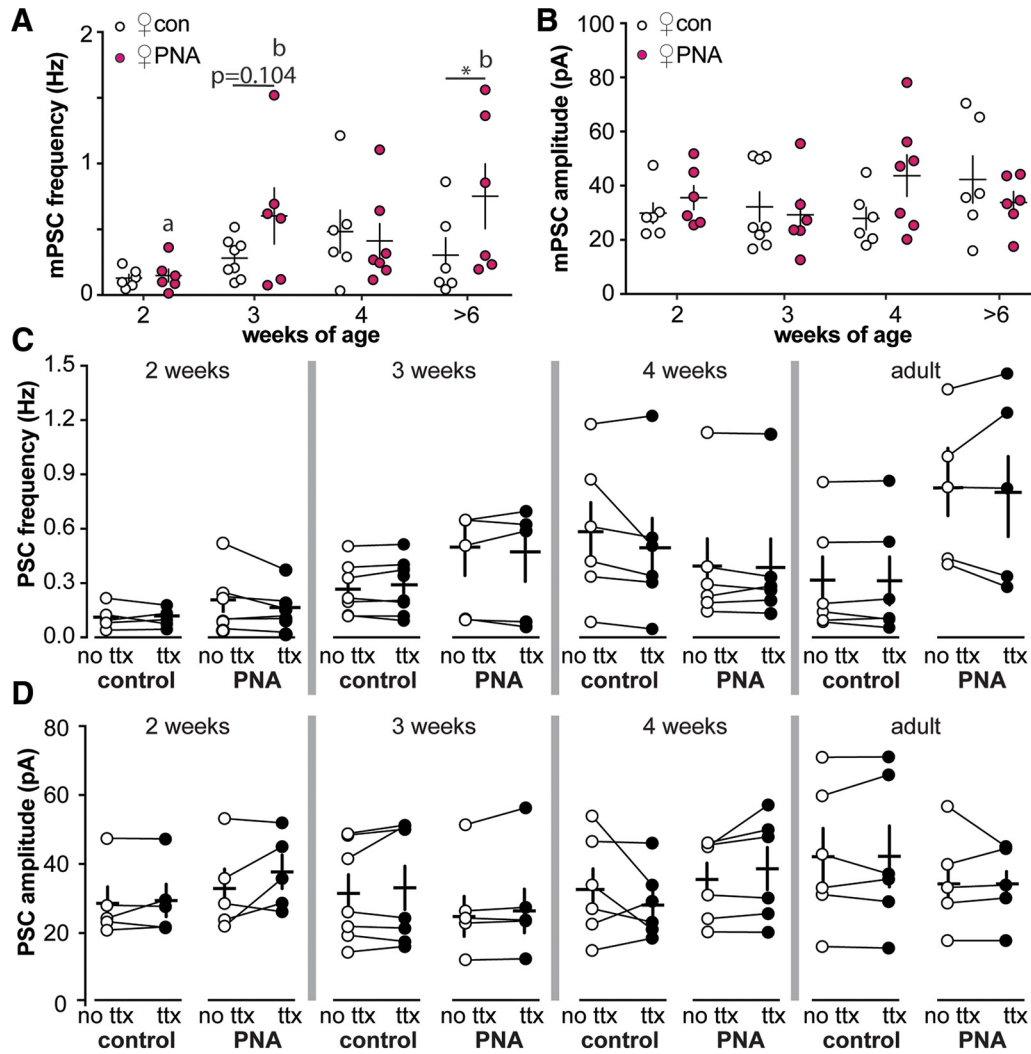


Figure 4. Activity-independent GABAergic transmission to GnRH neurons does not change throughout the prepubertal period or with PNA treatment in females. Individual values and mean \pm SEM (crosses) of mPSC frequency (**A**) and mPSC amplitude (**B**) at 2, 3, and 4 weeks and adults >6 weeks. Data for 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. * $p < 0.05$ between groups at each age. **C, D**, GABAergic PSC frequency (**C**) and amplitude (**D**) in cells under control conditions (no TTX, open circles) and in the presence of $1 \mu\text{M}$ 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.

Table 7. Kinetic parameters of GABA mPSCs in GnRH neurons

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

Values are the mean \pm SEM. No differences were observed among groups.

9 neurons) firing in response to GABA ($p < 0.05$ vs con) vs 83% of cells (5 of 6 cells) from 2-week-old PNA females and 71% of cells (5 of 7 cells) from 4-week-old PNA females (Fig. 5F). The percentage of firing cells was not different between PNA and control mice at 2 weeks of age ($p = 0.462$) or 4 weeks of age ($p > 0.99$). There were no changes in input resistance or capacitance with PNA that would contribute to the observed differences (Tables 2, 3).

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

GABA-induced membrane potential depolarization of GnRH neurons is blunted in cells from 3-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 whether PNA changes the 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

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

Factor	Age (weeks)	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.

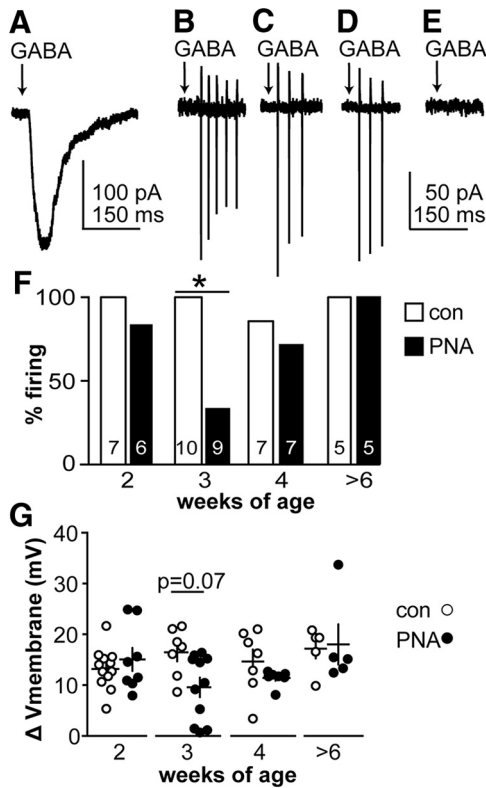


Figure 5. Response of GnRH neurons from PNA mice to acute GABA application is blunted at 3 weeks of age. **A**, Whole-cell voltage-clamp recording of current response to GABA in a GnRH neuron from an adult control female. **B–E**, Representative on-cell recordings of firing response (vertical action currents) to GABA in cells from adult control (**B**), adult PNA (**C**), 3-week-old control (**D**), and 3-week-old PNA female (**E**) mice. **A–E** are on the same time scale. **F**, Percentage of GnRH neurons that fire in response to GABA. The 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 \pm 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, $F_{(3,56)} = 2.1$).

Table 10. Baseline membrane potential of GnRH neurons

Age (weeks)	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, $n = 5$

Values are the mean \pm SEM. No differences were observed among groups; treatment, $F_{(1,84)} = 0.01$; age, $F_{(4,84)} = 0.07$; interaction, $F_{(3,58)} = 1.25$.

all GnRH neurons studied at all ages (Fig. 5G). In GnRH neurons from PNA females, however, the magnitude of depolarization was blunted at 3 weeks of age compared with controls ($p < 0.05$).

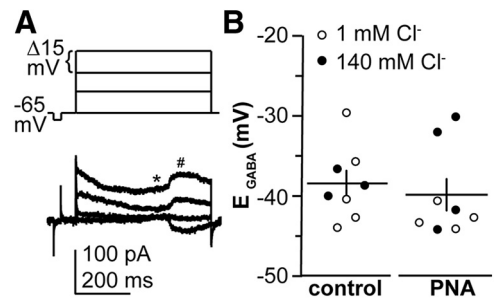


Figure 6. E_{GABA} is not altered by PNA in GnRH neurons from 3-week-old females. **A**, Top, Voltage step protocol from -65 to -15 mV. Bottom, Representative gramicidin perforated-patch recording showing the current response to voltage steps. * and # show where measurements were made of baseline and $GABA_A$ R-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 and 140 mM chloride pipette solutions, respectively. Data were analyzed using two-tailed, unpaired Student’s t test, $p = 0.7048$.

Reversal potential for $GABA_A$ receptor-mediated current is not altered by PNA in 3-week-old females

Gramicidin perforated-patch recordings were used to measure the reversal potential for the $GABA_A$ receptor-mediated current (E_{GABA} ; Fig. 6). E_{GABA} was not different between control and PNA mice at 3 weeks of age. No difference in E_{GABA} was observed between measures made using 1 vs 140 mM chloride pipette solutions, indicating that the measured reversal potentials were dependent on intrinsic chloride concentration and were not contaminated by the recording.

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 the effects of PNA treatment and sex on the 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 1 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 that 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; Andrae 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 3 weeks of age, a difference that was maintained in adulthood. This increase was activity independent, suggesting that 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 5 in females and prenatally in males (Pointis et al., 1979; Mannan and O'Shaughnessy, 1991); it is not known whether 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 3-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 3-week-old PNA mice receive increased GABA transmission compared with 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 3-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 3-week-old control and PNA females, suggesting that any such changes have minimal perisomatic functional impact. Second, PNA may alter 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 induce arterial vasodilation (Ding and Stallone, 2001; Cairrão 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 the amplitude or kinetics of either mPSCs or sPSCs, suggesting that changes in postsynaptic GABA_A receptors or the 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 the GnRH neuron response to GABA in 3-week-old PNA mice, these are apparently lost during development or become insufficient to reduce firing as there was no effect of PNA on the response to GABA in cells from adults. The loss of these adaptive mechanisms may be an important factor in upregulating GnRH neuron output in adult PNA mice and possibly in women with PCOS.

The effects of PNA are most likely dependent upon activation of androgen receptors, as the nonaromatizable androgen DHT was used. In the hippocampus, androgens increase synaptic spines (Brawer et al., 1983; Hajszan et al., 2007, 2008; Hatanaka et al., 2015) and synaptic transmission during pubertal development (Petrossi et al., 2013). Additionally, glia express androgen receptor and play key roles in synapse formation and regulation (García-Segura et al., 1994; Tasker et al., 2012). Within the hypothalamus, many kisspeptin neurons in the arcuate and anteroventral 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 anteroventral periventricular 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 the activation of estrogen receptor β (ER β) by DHT metabolites (Lund et al., 2006). ER β 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 the 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 with 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 sensitivities to programming and/or activation 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 a 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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