

Neuronal Excitability

GnRH Neuron Excitability and Action Potential Properties Change with Development But Are Not Affected by Prenatal Androgen Exposure

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Abstract

Gonadotropin-releasing hormone (GnRH) neurons produce the final output from the brain to control pituitary gonadotropin secretion and thus regulate reproduction. Disruptions to gonadotropin secretion contribute to infertility, including polycystic ovary syndrome (PCOS) and idiopathic hypogonadotropic hypogonadism. PCOS is the leading cause of infertility in women and symptoms resembling PCOS are observed in girls at or near the time of pubertal onset, suggesting that alterations to the system likely occurred by that developmental period. Prenatally androgenized (PNA) female mice recapitulate many of the neuroendocrine phenotypes observed in PCOS, including altered time of puberty, disrupted reproductive cycles, increased circulating levels of testosterone, and altered gonadotropin secretion patterns. We tested the hypotheses that the intrinsic properties of GnRH neurons change with puberty and with PNA treatment. Whole-cell current-clamp recordings were made from GnRH neurons in brain slices from control and PNA females before puberty at three weeks of age and in adulthood to measure GnRH neuron excitability and action potential (AP) properties. GnRH neurons from adult females were more excitable and required less current to initiate action potential firing compared with three-week-old females. Further, the afterhyperpolarization (AHP) potential of the first spike was larger and its peak was delayed in adulthood. These results indicate development, not PNA, is a primary driver of changes to GnRH neuron intrinsic properties and suggest there may be developmentally-induced changes to voltage-gated ion channels in GnRH neurons that alter how these cells respond to synaptic input.

Key words: development; electrophysiology; GnRH neurons; intrinsic properties; prenatal androgenization

Significance Statement

Gonadotropin-releasing hormone (GnRH) neurons play a crucial role in reproductive function. Disruptions to the release of pattern of GnRH secretion are implicated in fertility disorders, such as polycystic ovary syndrome (PCOS). Prenatally androgenized (PNA) female mice recapitulate many of the neuroendocrine phenotypes observed in women diagnosed with PCOS. We used electrophysiology to study how the intrinsic properties of GnRH neurons are altered with pubertal development and with PNA treatment. We found that prepubertal versus postpubertal GnRH neurons had different properties, including increased excitability after puberty. PNA treatment did not affect these typical developmental changes. These data suggest the postulate that development, rather than androgen exposure, is a primary regulator of the voltage-gated ion channels of GnRH neurons.

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Introduction

Gonadotropin-releasing hormone (GnRH) is the final output from the brain for the neuroendocrine control of reproduction. GnRH release occurs in an episodic manner from neurons located in the midventral preoptic area and hypothalamus (Clarke and Cummins, 1982; Moenter et al., 1992; Knobil and Neill, 1994). GnRH acts on the anterior pituitary to stimulate synthesis and release of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH); low-frequency GnRH pulses favor these processes for FSH whereas high-frequency GnRH pulses favor LH (Wildt et al., 1981; Haisenleder et al., 1991). In females, FSH and LH regulate ovarian follicle maturation and steroidogenesis. Disruptions to gonadotropin secretion patterns, implying altered GnRH release, can contribute to infertility including idiopathic hypothalamic hypogonadism (Legro, 2003; Tsutsumi and Webster, 2009; Sidhoum et al., 2014) and polycystic ovary syndrome (PCOS; Legro, 2003; Burt Solorzano et al., 2012). PCOS is the leading cause of infertility in females, affecting up to 20% of females of reproductive age according to the Rotterdam criteria, for which patients must exhibit at least two of three symptoms: hyperandrogenemia, oligo/anovulation, and/or polycystic ovarian morphology (Chang and Katz, 1999; Legro, 2003; McCartney and Marshall, 2016). Hyperandrogenemic PCOS is diagnosed in 8–10% of all women and gonadotropin release in these women is characterized by persistently high LH pulse frequency and elevated LH/FSH ratio (Taylor et al., 1997; McCartney et al., 2002; Patel et al., 2004; Tsutsumi and Webster, 2009), suggesting at least some changes are occurring centrally at the level of GnRH release.

To study underlying central mechanisms, we must turn to animal models that recapitulate aspects of PCOS (Walters et al., 2018). In several species, including primates, mice, rats and sheep, *in utero* exposure to androgens produces offspring that develop reproductive neuroendocrine phenotypes similar to hyperandrogenemic PCOS (Levine et al., 1985; Robinson et al., 2002; Sullivan and Moenter, 2004; Foecking et al., 2005; Mahoney and Padmanabhan, 2010; Roland and Moenter, 2011; Witham et al., 2012; Moore et al., 2015; Abbott et al., 2016; Coyle and Campbell, 2019). In adulthood, prenatally androgenized (PNA) mice exhibit disrupted estrous cycles, elevated LH pulse frequency and increased testosterone levels compared with controls (Roland and

Moenter, 2011; Moore et al., 2015; Dulka and Moenter, 2017; Berg et al., 2018). GnRH neurons from adult PNA mice have a higher action potential (AP) firing rate (Roland and Moenter, 2011; Dulka and Moenter, 2017). This elevated firing rate is likely driven at least in part by PNA-induced increases in GABAergic transmission to these cells (Sullivan and Moenter, 2004; Berg et al., 2018); unlike most neurons, GABA signaling via the GABA_A receptor can induce action potential firing by GnRH neurons (DeFazio et al., 2002; Herbison and Moenter, 2011). Whether or not intrinsic changes in GnRH neurons also contribute to the increased firing rate of these cells in adult PNA animals is not known.

The onset of PCOS is thought to occur near puberty (Ibañez et al., 1993; Franks, 2002; Rosenfield, 2007; Ibañez et al., 2009; Burt Solorzano et al., 2010) and studies in PNA mice suggest neurobiological changes are already occurring before puberty is complete. Specifically, in contrast to the increased firing rate observed in GnRH neurons from PNA adults, GnRH neurons from three-week-old PNA mice have reduced firing rates compared with controls (Roland and Moenter, 2011; Dulka and Moenter, 2017). Interestingly, GnRH neurons from three-week-old PNA mice still receive increased GABAergic transmission compared with controls (Berg et al., 2018). The decrease in GnRH neuron activity in combination with the increase in GABA transmission to these cells in three-week-old PNA mice was surprising because we would expect the increase in GABA drive to increase firing activity unless the response to GABA is altered. Indeed, PNA treatment decreases the percentage of GnRH neurons from three-week-old PNA mice that increase firing in response to locally-applied GABA, suggesting their response to GABA is indeed altered. This was not attributable to changes in either baseline membrane potential or the reversal potential for GABA_A-mediated current (Berg et al., 2018), indicating that either other elements of GABA_A receptor signaling, for example receptor composition, and/or changes in other intrinsic properties of GnRH neurons contribute to the reduced ability of GABA to induce action potential firing in GnRH neurons from prepubertal PNA mice.

Here, we began to characterize the response of GnRH neurons to current injection to examine how development and/or PNA treatment alter intrinsic properties of these cells. Specifically, we examined the excitability of GnRH neurons, defined as the number of action potentials generated as a function of current injected, and their response to hyperpolarizing current injections. By performing these studies in both control and PNA mice at three weeks of age and in adulthood, we gained valuable information about the normal development of GnRH neuron excitability during the pubertal process, and how PNA treatment affects this developmental trajectory. We hypothesized that GnRH neurons from PNA mice are less excitable at three weeks of age compared with controls, but become more excitable than controls during adulthood.

Materials and Methods

All chemicals were acquired from Sigma-Aldrich unless noted.

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Animals

GnRH-GFP (Tg(Gnrh1-EGFP)51Sumo MGI:6158457) mice (Suter et al., 2000) were bred in our colony. All mice were provided with water and Harlan 2916 (nonbreeders) or 2919 (breeders) chow *ad libitum* and were held on a 14/10 h light/dark cycle with lights on at 3 A.M. Eastern Standard Time. To generate PNA mice, female GnRH-GFP transgenic mice on a C57Bl/6J background and a CD1 female were bred with a C57Bl/6J male and monitored daily for a copulatory plug (day 1 of pregnancy). The CD1 dam assists in providing maternal care and nutrition. On days 16–18 of pregnancy, GnRH-GFP dams were injected subcutaneously with 225 μ g/d of dihydrotestosterone (DHT) for PNA or sesame oil for vehicle controls. Combined litter sizes were adjusted to <15 pups by culling CD1 pups to standardize nutrition. Adult female mice were studied on the morning of diestrus, determined via vaginal cytology and uterine mass. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Brain slice preparation

All solutions were bubbled with 95% O₂/5% CO₂ for at least 15 min before use with tissue and throughout the experimental recordings. Brain slices were prepared 4–8.5 h after lights on as described (DeFazio and Moenter, 2002). Brains were 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 (350 mOsm). Coronal slices (300 μ m) through the hypothalamic region were cut with a Leica VT1200S Microtome (Leica Biosystems). Slices were incubated for 30 min at room temperature (~21–23°C) in 50% sucrose saline and 50% artificial CSF (ACSF; containing (in mM): 135 NaCl, 3.5 KCl, 26 NaHCO₃, 10 D-glucose, 1.25 Na₂HPO₄, 1.2 MgSO₄, and 2.5 CaCl₂, 315 mOsm, pH 7.4). Slices were then transferred to 100% ACSF solution at room temperature for 0.5–5 h before recording. A minimum of five mice from at least four litters were studied per group; up to four recordings were used per mouse.

Recording solutions and data acquisition

Whole-cell patch-clamp recordings in current-clamp mode were conducted to investigate the intrinsic properties of GnRH neurons from PNA and control mice before and after puberty. The pipet solution contained (in mM): 125 K gluconate, 20 KCl, 10 HEPES, 5 EGTA, 0.1 CaCl₂, 4 MgATP, and 0.4 NaGTP, 305 mOsm, pH 7.2 with NaOH. This solution is based on the native intracellular chloride concentrations in GnRH neurons determined using gramicidin perforated-patch recordings (DeFazio et al., 2002; Berg et al., 2018). A 14.5-mV liquid junction potential was negated online before each recording. During recordings, brain slices were continuously perfused with carboxygenated ACSF (3 ml/min) and kept at 30–31°C with an inline-heating unit (Warner Instruments Model SH-27B). GFP-positive GnRH neurons were visualized with infrared differential interference contrast and fluorescence microscopy on an

Olympus BX51WI microscope. All recordings were made using an EPC-10 patch-clamp amplifier and a computer running PatchMaster software (HEKA Elektronik). To monitor recording quality, input resistance, series resistance, baseline current, and capacitance were monitored throughout experiments from the averaged membrane current response to 16 hyperpolarizing voltage steps from –70 mV (5 mV, 20 ms, acquisition 100 kHz, filter 10 kHz). Data were analyzed using IgorPro (Wavemetrics). Only recordings with input resistance of >500 M Ω , stable compensated series resistance of <20 M Ω and a stable capacitance were used for analysis.

Experimental design

GnRH neuron excitability, the number of action potentials generated as a function of current injection, and action potential properties were monitored in GnRH neurons in the preoptic area of brain slices from prepubertal (18–21 d) and adult (84–130 d) gonad-intact control and PNA female mice; adults were studied in diestrus, determined by vaginal cytology. PNA status was confirmed by monitoring age at vaginal opening (VO), estrous cyclicity by vaginal lavage for 14 consecutive days, and measuring anogenital distance (AGD) between 70 and 84 d of age (mean of three successive daily measures).

Excitability and action potential property analysis

To characterize GnRH neuron excitability and action potential properties, current-clamp recordings were obtained (20-kHz acquisition, 10-kHz filter) in the presence of ionotropic glutamate and GABA receptor antagonists [20 μ M D-APV (Tocris), 10 μ M CNQX (Tocris), 100 μ M picrotoxin]. Bridge balance (95%) was used for all experiments. Cells were maintained within 1.5 mV of –70 mV. Basal membrane potential was calculated during the 1 ms before the start of current injection. Current steps (500 ms, 5-pA increments from 0 to +40 pA, 5-pA decrements from 0 to –50 pA) were delivered to test the membrane potential response. The first action potential (AP) observed at the rheobase, the minimum current needed to induce action potentials, was analyzed in detail. Action potential threshold was defined as the potential at which the membrane potential slope exceeded 1 V/s. Action potential latency was the time from start of the current injection to threshold. Rate of rise was the maximum voltage derivative from threshold to peak. Full width of the action potential at half maximum (FWHM) between threshold and peak, and after-hyperpolarization (AHP) time and amplitude relative to threshold were also calculated. The membrane response to hyperpolarizing current injection was quantified as the difference between the average membrane potential over 1 ms around the peak sag potential and the average membrane potential over 1 ms at the peak steady state potential. The membrane response of GnRH neurons following the termination of the hyperpolarizing current injection was quantified as the difference between the average of membrane potential for 1 ms around the peak repolarization potential and the basal membrane potential before current injection.

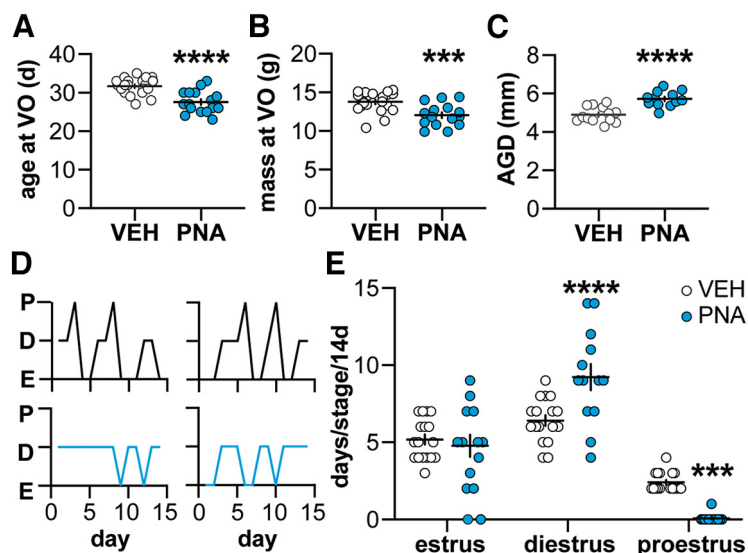


Figure 1. Confirmation of PNA phenotype. **A–C**, Individual values and mean \pm SEM for age of vaginal opening (VO; **A**), body mass at VO (**B**), and adult anogenital distance (AGD, mm; **C**). **D**, Representative estrous cycles over 14 d. P, proestrus; D, diestrus; E, estrus. **E**, Individual values and mean \pm SEM days in each cycle stage over 14 d. Statistical parameters are in Table 1; *** $p < 0.005$, **** $p < 0.0001$.

Statistics

Data are reported as mean \pm SEM, with individual values shown when practical. Statistical analyses were made using Prism 9 (GraphPad Software). Data were tested for normal distribution with Shapiro–Wilk. Details of specific tests are provided in the results. Statistical tables for two-way ANOVAs report the differences in means and associated 95% confidence interval (CI) defined for age (three-week-adult), treatment (control-PNA), and interaction [(adult-CON – adult-PNA) – (three-week CON – three-week PNA)]; α was set to 0.05 and n indicates the number of mice for Figure 1 and number of cells for electrophysiology.

Results

Verification of prenatal androgenization phenotype

PNA-induced differences were confirmed in the present study; for mice in which electrophysiology was done at three weeks of age and in adulthood, these aspects were verified in littermates raised to adults as the PNA phenotype is consistent among littermates. As reported (Roland and Moenter, 2011; Dulka and Moenter, 2017; Berg et al., 2018; Gibson et al., 2021), vaginal opening (VO) in the present study occurred at a younger age (unpaired, two-tailed Student's t test; control $n = 19$ mice, PNA $n = 16$ mice) and lower body mass (two-tailed Mann–Whitney U test) in PNA females (Fig. 1*A,B*; Table 1). Anogenital distance (AGD) was increased in adult PNA mice (unpaired, two-tailed Student's t test; Fig. 1*C*; Table 1; control $n = 15$ mice, PNA $n = 17$ mice) and estrous cycles were disrupted. Specifically, PNA females spent fewer days in proestrus and more days in diestrus (χ^2 , Fig. 1*D,E*; Table 1; control $n = 17$ mice, PNA $n = 17$ mice).

Recording quality parameters and passive properties of GnRH neurons

Passive properties and series resistance were used to assess recording quality. There were no differences among

these parameters between depolarizing and hyperpolarizing current injection protocols, and they were combined for quality assessment (two-way ANOVA; three-week control $n = 15$ cells, three-week PNA $n = 18$ cells, adult control $n = 15$ cells, adult PNA $n = 17$). There were no differences in compensated series resistance or capacitance among groups (Fig. 2*A,B*; Table 2). Input resistance was greater and holding current was more hyperpolarized in cells from adult than three-week-old mice (Fig. 2*C,D*; Table 2).

GnRH neurons from adult females are more excitable than those from three-week-old females

Whole-cell current-clamp recordings were used to assess excitability measured as the number of action potentials generated as a function of current injection. Figure 3*A* shows representative membrane potential traces (top) in response to current injection (bottom) for each group; only three steps are shown for clarity. GnRH neurons from adult mice generated more action potentials in response to depolarizing current than those from three-week-old females, but PNA treatment had no effect on excitability (three-way, repeated-measures ANOVA, Fig. 3*B*; Table 3; three-week control $n = 14$ cells, three-week PNA $n = 12$ cells, adult control $n = 12$ cells, adult PNA $n = 17$ cells). Based on results of the three-way ANOVA, data were consolidated by age or treatment for comparison and re-analyzed by two-way, repeated-measures ANOVA, which found a difference for age and thus was followed by Bonferroni *post hoc* (Fig. 3*B*; Table 3). Age, but not treatment, consolidated data show that GnRH neurons from adult mice fire more action potentials in response to depolarizing current than those from three-week-old females.

Developmental stage alters GnRH neuron action potential properties

To generate hypotheses about specific ionic current changes that may underlie the above observations, we

Table 1: Descriptive statistics and statistical parameters characterizing the PNA phenotype (Fig. 1)

Mean ± SEM for age at VO, body mass at VO and AGD			
Property	VEH		PNA
Age at vaginal opening (d)	31.7 ± 0.5		27.6 ± 0.7
Body mass (g) at vaginal opening	13.8 ± 0.3		12.1 ± 0.4
AGD (mm)	4.9 ± 0.1		5.7 ± 0.1
Property	Unpaired, two-tailed Student's <i>t</i> test	Mean difference (PNA-VEH)	Effect size <i>r</i> ²
Age at vaginal opening (d)	<i>t</i> _(4.986) , df = 34; <i>p</i> < 0.0001	Diff [CI, −5.824, −2.451] −4.138 ± 0.8299	<i>r</i> ² = 0.4223
AGD (mm)	<i>t</i> _(5.124) , df = 23; <i>p</i> < 0.0001	Diff [CI, 0.8206, 0.1601] 0.8206 ± 0.160	<i>r</i> ² = 0.5331
Property	Mann–Whitney <i>U</i> test		Two-tailed <i>p</i> -value
Body mass (g) at vaginal opening	<i>U</i> = 55		<i>p</i> = 0.0006
Mean ± SEM days per estrous cycle stage			
Cycle stage	VEH		PNA
Estrus	5.2 ± 0.3		4.5 ± 0.7
Diestrus	6.4 ± 0.3		9.2 ± 0.8
Proestrus	2.4 ± 0.2		0.1 ± 0.1
Property	χ^2 test		
Estrous cycle stage distribution	χ^2 = 36.1672, <i>n</i> = 425, df = 2, <i>p</i> = 0.000000014		
	Estrus	Diestrus	Proestrus
Std. residual	0.3578012	3.771665	5.7238930
Fisher's exact test; Bonferroni adjusted	<i>p</i> = 1.0	<i>p</i> = 0.000573	<i>p</i> < 0.00005

Bold indicates $p < 0.05$.

examined properties of the first spike generated at rheobase (Tables 4, 5). Representative traces aligned by AP threshold are shown in Figure 3C for each group. There were no differences in AP threshold, latency, rate of rise, amplitude, or FWHM among groups (two-way ANOVA; Fig. 3D–H). The rheobase was lower in cells from adult mice, consistent with the increased excitability and input resistance (Fig. 3I). Despite the similarity of the spike phase of the AP, the AHP differed among groups. Specifically, the AHP peaked later in adult compared with three-week-old females regardless of PNA treatment (Fig. 3J), and the amplitude approached the level

set for significance for being larger in adults (Fig. 3K). Together, these observations suggest that changes in the action potential properties of GnRH neurons are primarily driven by development.

There are no differences in the response of GnRH neurons to hyperpolarizing current during development or with PNA treatment

Representative membrane voltage traces (top) in response to the hyperpolarizing current are shown in Figure 4A; only three current steps are shown for

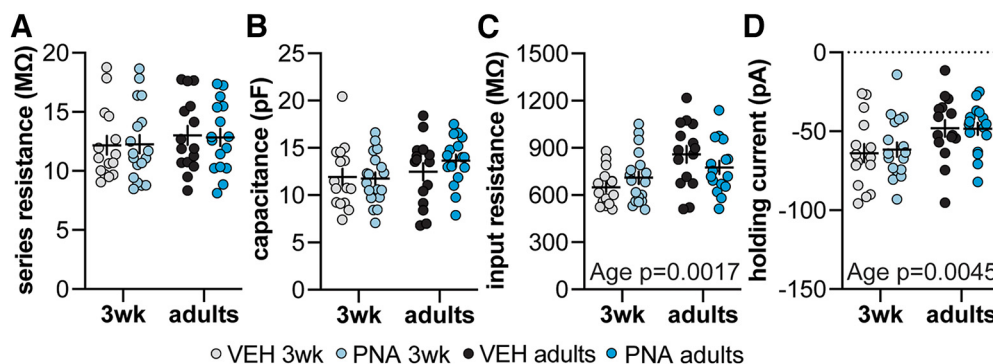


Figure 2. Recording quality parameters. **A–D**, Individual values and mean \pm SEM for compensated series resistance (**A**), capacitance (**B**), input resistance (**C**), holding current (**D**). Statistical parameters are in Table 2.

Table 2: Descriptive statistics and statistical parameters from two-way ANOVA for recording quality parameters and passive properties (Fig. 2)

Descriptive statistics (mean ± SEM)				
Property	3-week controls	3-week PNA	Adult controls	Adult PNA
Series resistance (MΩ)	12.2 ± 0.8	12.259 ± 0.8	13.0 ± 0.8	12.841 ± 0.729
Capacitance (pF)	11.9 ± 0.9	11.8 ± 0.6	12.5 ± 0.9	13.654 ± 0.651
Input resistance (MΩ)	648.6 ± 30.2	710.3 ± 39.3	858.6 ± 53.4	775.946 ± 41.81
Holding current (pA)	−63.9 ± 5.8	−61.6 ± 4.6	−48.2 ± 5.3	−48.456 ± 3.820

Property	Age	Two-way ANOVA			
		Treatment		Interaction	
Series resistance (MΩ)	Diff, −0.7058 [CI, −2.250, 0.8387] $F_{(1,60)} = 0.8356$; $p = 0.3643$	Diff, 0.04112 [CI, −1.503, 1.586] $F_{(1,60)} = 0.002837$; $p = 0.9577$		Diff, −0.2489 [CI, −2.840, 3.338] $F_{(1,60)} = 0.02597$; $p = 0.8725$	
Capacitance (pF)	Diff, −1.220 [CI, −2.750, 0.3089] $F_{(1,60)} = 2.548$; $p = 0.3906$	Diff, −0.5158, [CI, −2.045, 1.014] $F_{(1,60)} = 0.4551$; $p = 0.5025$		Diff, 1.322 [CI, −4.381, 1.736] $F_{(1,60)} = 0.7478$; $p = 0.5025$	
Input resistance (MΩ)	Diff, −137.8 [CI, −221.8, −53.87] $F_{(1,60)} = 10.78$; $p = 0.0017$ Bonferroni	Diff, 10.43 [CI, −73.53, 94.39] $F_{(1,60)} = 0.06174$, $p = 0.8046$		Diff, −144.4 [CI, −312.3, 23.56] $F_{(1,60)} = 2.957$; $p = 0.0907$	
Holding current (pA)	Diff, −14.42 [CI, −24.17, −4.658] $F_{(1,60)} = 8.732$; $p = 0.0045$ Bonferroni	3-week VEH	3-week VEH	3-week PNA	Adult VEH
		vs 3-week PNA	vs adult VEH	vs adult PNA	vs adult PNA
		$p > 0.9999$	$p = 0.0065$	$p \geq 0.9999$	$p > 0.9999$
		Diff, −0.9966 [CI, −10.75, 8.761] $F_{(1,60)} = 0.04174$; $p = 0.8388$		Diff, −2.608 [CI, −22.12, 16.91] $F_{(1,60)} = 0.07146$; $p = 0.7901$	
		3-week VEH	3-week VEH	3-week PNA	Adult VEH
		vs 3-week PNA	vs adult VEH	vs adult PNA	vs adult PNA
		$p > 0.9999$	$p = 0.1846$	$p = 0.3271$	$p > 0.9999$

Bold indicates $p < 0.05$.

clarity. No cells exhibited rebound spikes in response to termination of hyperpolarizing current injection. To assess membrane sag typically associated with activation of hyperpolarization-activated current (I_h), comparisons were made among cells that were hyperpolarized to a membrane potential between −90 and −95 mV. Negative current between −10 to −45 pA was needed to hyperpolarize GnRH neurons to this membrane potential range and less hyperpolarizing current was required to achieve this membrane potential in adulthood compared with three-week-old females regardless of PNA treatment (Table 6; three-week control $n = 11$ cells, three-week PNA $n = 10$ cells, adult control $n = 11$ cells, adult PNA $n = 13$ cells). The membrane potential difference between peak of the sag and steady-state (sag) was both minimal and similar among experimental groups (Fig. 4B; Table 6). There was also no difference in GnRH neuron rebound depolarization after termination of the current injection (Fig. 4C; Table 6). These data suggest neither development nor PNA treatment alter the response of GnRH neurons to hyperpolarizing input within the range tested.

Discussion

Reproduction is centrally controlled by the release pattern of GnRH. Disruptions in GnRH release are postulated to occur in hyperandrogenemic women with PCOS based on the observed increased frequency of pulsatile LH secretion. We used a mouse model that recapitulates the

neuroendocrine aspects of PCOS to test the hypotheses that intrinsic properties of GnRH neurons change during typical development, and that these developmental changes are altered by PNA treatment (Roland and Moenter, 2011; Moore et al., 2015; Dulka and Moenter, 2017; Berg et al., 2018). Data supported the first hypothesis but rejected the second as the intrinsic properties of GnRH neurons tested change with age but are not affected by PNA treatment. Specifically, GnRH neurons from adult mice are more excitable and have altered action potential properties compared with those from three-week-old mice.

During typical development, the spontaneous action potential firing rate of GnRH neurons is dynamic, with mean GnRH neuron firing rate peaking at three weeks of age before decreasing to lower levels in adulthood (Roland and Moenter, 2011; Dulka and Moenter, 2017). In contrast, the firing rate in GnRH neurons from PNA mice is steady throughout development. As a result, firing rate of GnRH neurons from PNA mice is lower than controls at three weeks of age, but increased in adulthood (Roland and Moenter, 2011; Dulka and Moenter, 2017). These observations indicate that both development and PNA treatment play a role in shaping the activity of these neurons. This overall firing rate is shaped by a combination of the intrinsic properties of and synaptic inputs to GnRH neurons, as well as interactions with non-neuronal cells like glia (Prevot and Sharif, 2022). The present work suggests that the intrinsic properties of these neurons are developmentally regulated but not affected by PNA treatment.

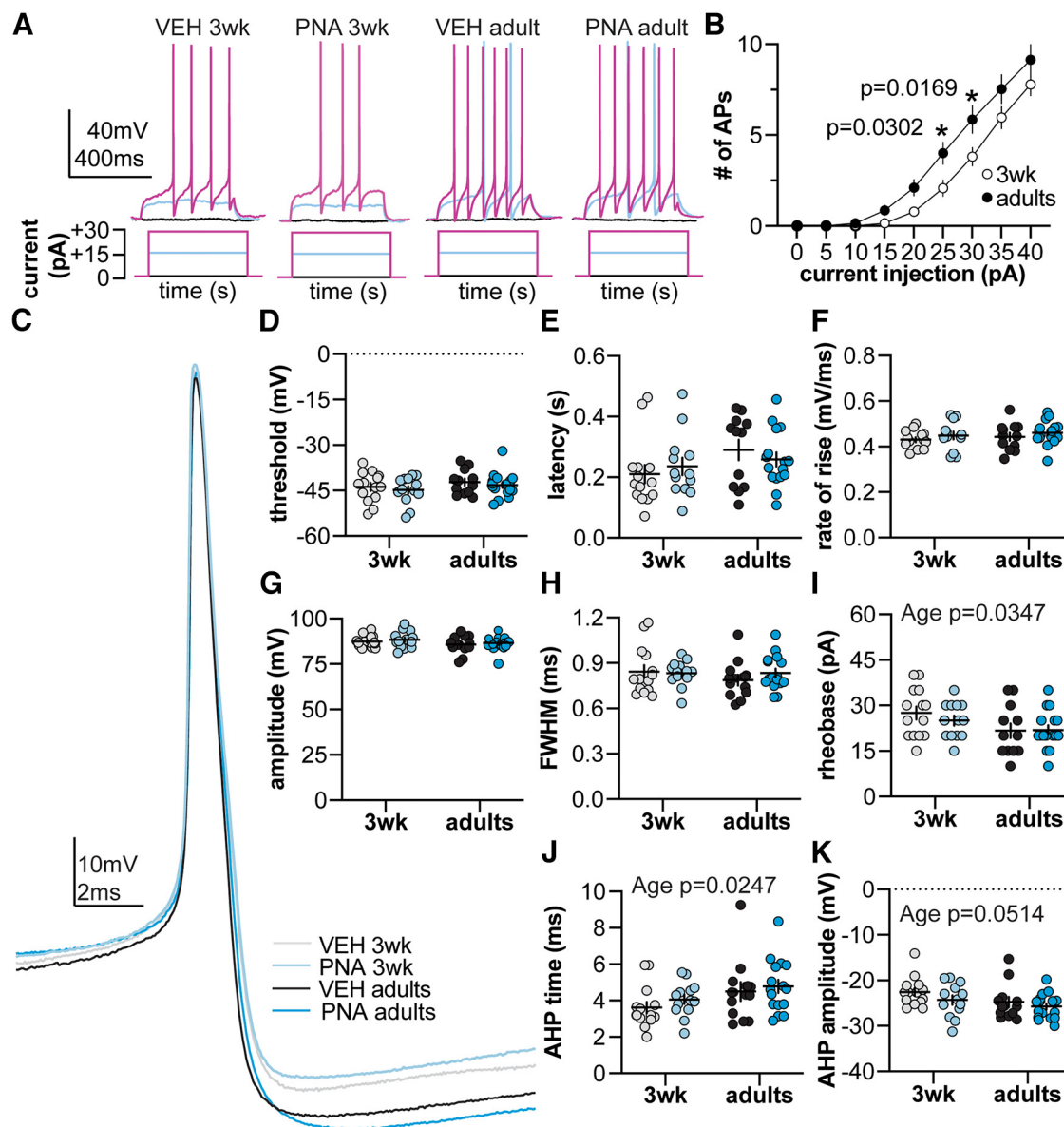


Figure 3. GnRH neuron excitability is increased, and action potential properties altered, in adult versus three-week-old mice. **A**, Representative membrane voltage responses (top) to depolarizing current injections (bottom); only three current steps are shown for clarity. **B**, Mean \pm SEM # of action potentials (APs) fired as a function of current injection in age-combined groups. **C**, representative traces of the rheobase AP for each experimental group. **D–K**, Individual values and mean \pm SEM for AP threshold (**D**), latency (**E**), rate of rise (**F**), AP amplitude (**G**), full width at half-maximum (FWHM; **H**), rheobase (**I**), afterhyperpolarization potential (AHP) time (**J**), and AHP amplitude (**K**). Statistical parameters are in Tables 3 and 4.

This finding is consistent with work in which transcriptome profiling of GnRH neurons from the same groups revealed more developmental changes than changes that were induced by PNA treatment (Burger et al., 2020). Whereas GnRH neuron membrane response to depolarizing current changed between the ages studied, there were no differences among groups in membrane response to hyperpolarizing current or in rebound firing.

To begin to formulate hypotheses about the changes that might underlie the observed postpubertal increase in GnRH neuron excitability, properties of the first action potential were measured. In adulthood, GnRH neurons also

have increased input resistance, which likely is a strong contributor to two other changes observed with development, specifically increased excitability and reduced amount of current needed to induce firing (rheobase). Cells from adults also had a larger and delayed peak of the afterhyperpolarization potential regardless of PNA treatment. Changes to voltage-gated channels likely underlie these developmental differences. Potassium currents are major regulators of neuronal excitability and also sculpt the afterhyperpolarization potential. Blocking 4-aminopyridine-sensitive potassium channels in dorsal root ganglion neurons decreases the latency to fire in response to current

Table 3: Descriptive statistics and statistical parameters from three-way repeated-measures ANOVA for GnRH neuron excitability (Fig. 3)

Current (pA)	Descriptive statistics number of APs (mean \pm SEM)			
	3-week controls	3-week PNA	Adult controls	Adult PNA
0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
5	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
10	0.0 \pm 0.0	0.0 \pm 0.0	0.2 \pm 0.12	0.1 \pm 0.13
15	0.1 \pm 0.14	0.2 \pm 0.15	1.1 \pm 0.50	0.7 \pm 0.30
20	0.8 \pm 0.35	0.8 \pm 0.40	2.3 \pm 0.75	2.0 \pm 0.56
25	2.3 \pm 0.70	1.8 \pm 0.56	4.3 \pm 1.09	3.8 \pm 0.71
30	3.6 \pm 0.84	4.0 \pm 0.57	6.2 \pm 1.36	5.6 \pm 0.88
35	5.9 \pm 1.10	6.1 \pm 0.63	8.0 \pm 1.45	7.2 \pm 0.90
40	7.7 \pm 1.00	7.8 \pm 0.68	9.7 \pm 1.51	8.8 \pm 0.94
Three-way repeated-measures ANOVA				
Current (pA)	$F_{(8,408)} = 176.9$; $p < 0.0001$	$F_{(1,341,71.06)} = 183.1$; $p < 0.0001$	$F_{(1,348,71.46)} = 173.9$; $p < 0.0001$	
Age	$F_{(1,51)} = 4.515$; $p = 0.0385$	$F_{(1,53)} = 4.478$; $p = 0.0391$		
Treatment	$F_{(1,51)} = 0.1273$; $p = 0.7227$		$F_{(1,53)} = 0.02802$; $p = 0.8677$	
Current \times age	$F_{(8,408)} = 2.977$; $p = 0.0030$	$F_{(8,424)} = 2.974$; $p = 0.0030$		
Current \times treatment	$F_{(8,408)} = 0.120$; $p = 0.9984$		$F_{(8,424)} = 0.06764$; $p = 0.9998$	
Age \times treatment	$F_{(1,51)} = 0.1738$; $p = 0.1738$			
Current \times age \times treatment	$F_{(8,408)} = 0.228$; $p = 0.9857$			
Bonferroni of age-consolidated data		Three weeks (CON and PNA) vs adult (CON and PNA)		
0, 5, 10, 15 pA		$p > 0.9999$		
20 pA		$p = 0.3820$		
25 pA		$p = 0.0302$		
30 pA		$p = 0.0169$		
35 pA		$p = 0.1481$		
40 pA		$p = 0.3349$		

Bold indicates $p < 0.05$.

injection and increases firing frequency (Vydyanathan et al., 2005). In motor neurons derived from patients with amyotrophic lateral sclerosis, partial pharmacological block of voltage-gated potassium currents reduces neuronal hypoexcitability and restores typical firing patterns (Naujock et al., 2016), and in cerebellar granule cells, a transient A-type potassium current increases during development concomitant with an increase in rheobase and spike latency (Shibata et al., 2000).

Similar effects of voltage-gated potassium conductances on excitability have been observed in hypothalamic neurons regulating reproduction. Estradiol-dependent increases in the membrane response to GABA and AMPA conductances applied with dynamic clamp in arcuate kisspeptin neurons were attributable to reduced A-type potassium currents (DeFazio et al., 2019). Consistent with

this, increasing the A-type potassium current in these neurons with dynamic clamp made firing irregular and increased the interval between action potentials (Mendonça et al., 2018). In GnRH neurons from adults, the A-type potassium current is reduced in a model of estradiol positive feedback (ovariectomized plus estradiol) compared with cells from ovariectomized mice (DeFazio and Moenter, 2002). This was associated with a reduced latency to action potential firing (DeFazio and Moenter, 2002) and may contribute to the increase in spontaneous firing rate observed during positive feedback (Christian et al., 2005; Silveira et al., 2017). These observations, along with the delayed AHP, increased AHP amplitude and differences in rheobase and excitability observed in the current study suggest that there may be developmental changes to potassium currents in GnRH neurons.

Table 4: Descriptive statistics for action potential properties (Fig. 3)

Property	Descriptive statistics (mean \pm SEM)			
	3-week controls	3-week PNA	Adult controls	Adult PNA
Threshold (mV)	-43.8 \pm 1.31	-44.8 \pm 1.24	-42.2 \pm 1.17	-43.2 \pm 0.95
Latency (s)	0.2 \pm 0.11	0.2 \pm 0.10	0.3 \pm 0.12	0.3 \pm 0.10
Amplitude (mV)	87.5 \pm 0.84	88.4 \pm 1.28	85.7 \pm 1.46	86.7 \pm 1.05
FWHM (ms)	0.8 \pm 0.04	0.8 \pm 0.02	0.8 \pm 0.04	0.8 \pm 0.03
Rate of rise (mV/ms)	0.4 \pm 0.01	0.4 \pm 0.02	0.4 \pm 0.02	0.5 \pm 0.01
Rheobase (mV)	27.5 \pm 2.15	25.0 \pm 1.60	21.7 \pm 2.41	21.9 \pm 1.57
AHP amplitude (mV)	-22.6 \pm 0.85	-24.3 \pm 1.02	-24.8 \pm 1.15	-25.8 \pm 0.68
AHP time (ms)	3.6 \pm 0.31	4.1 \pm 0.26	4.5 \pm 0.52	4.8 \pm 0.36

Table 5: Two-way ANOVA parameters for action potential properties (Fig. 3)

Property	Age	Treatment	Interaction
Threshold (mV)	Diff, 1.170 [CI, -3.918, 0.7768] $F_{(1,52)} = 1.803$; $p = 0.1852$	Diff, 0.9859 [CI, -1.361, 3.333] $F_{(1,52)} = 0.7103$; $p = 0.4032$	Diff, -0.01028 [CI, -4.705, 4.684] $F_{(1,52)} = 1.932e-005$; $p = 0.9965$
Latency (s)	Diff, -0.05101 [CI, -0.1089, 0.006901] $F_{(1,51)} = 0.3.127$; $p = 0.0.0830$	Diff, 0.003030 [CI, -0.05489, 0.06095] $F_{(1,51)} = 0.0.01103$; $p = 0.9168$	Diff, -0.05693 [CI, -0.1728, 0.05890] $F_{(1,51)} = 0.9735$; $p = 0.3285$
Amplitude (mV)	Diff, 1.731 [CI, -0.5921, 4.055] $F_{(1,51)} = 2.238$; $p = 0.1408$	Diff, -0.9231 [CI, -3.247, 1.400] $F_{(1,51)} = 0.0.6361$; $p = 0.4288$	Diff, 0.002851 [CI, -4.650, 4.644] $F_{(1,51)} = 1.517e006$; $p = 0.9990$
FWHM (ms)	Diff, 0.02731 [CI, -0.03989, 0.09454] $F_{(1,51)} = 0.6660$; $p = 0.4183$	Diff, -0.01879 [CI, -0.8601, 0.04843] $F_{(1,51)} = 0.3149$; $p = 0.5771$	Diff, 0.05826 [CI, -0.07618, 0.1927] $F_{(1,51)} = 0.7568$; $p = 0.3884$
Rate of rise (mV/ms)	Diff, -0.01261 [CI, -0.04178, 0.01657] $F_{(1,51)} = 0.7524$; $p = 0.3898$	Diff, 0.01453 [CI, -0.04740, 0.01095] $F_{(1,51)} = 0.2155$; $p = 0.2155$	Diff, -0.0002991 [CI, -0.05865, 0.05805] $F_{(1,51)} = 0.0001059$; $p = 0.9918$
Rheobase (pA)	Diff, 4.479 [CI, 0.5934, 8.365] $F_{(1,51)} = 5.355$; $p = 0.0247$ Bonferroni	Diff, 1.146 [CI, -2.740, 5.032] $F_{(1,51)} = 0.3505$; $p = 0.5565$ 3-week VEH vs 3-week PNA $p > 0.9999$	Diff, 2.708 [CI, -5.063, 10.48] $F_{(1,51)} = 0.4895$; $p = 0.4873$ 3-week PNA vs adult VEH $p > 0.9999$
AHP amplitude (mV)	Diff, 1.827 [CI, -0.01161, 3.666] $F_{(1,51)} = 3.980$; $p = 0.0514$ Bonferroni	Diff, 1.369 [CI, -0.4701, 3.208] $F_{(1,51)} = 2.233$; $p = 0.1412$ 3-week VEH vs 3-week PNA $p > 0.9999$	Diff, 0.7832 [CI, -2.895, 4.461] $F_{(1,51)} = 0.1827$; $p = 0.6708$ 3-week PNA vs adult VEH $p > 0.9999$
AHP time (ms)	Diff, -0.7990 [CI, -1.538, -0.05992] $F_{(1,51)} = 4.710$; $p = 0.0347$ Bonferroni	Diff, -0.3644 [CI, -1.103, 0.3747] $F_{(1,51)} = 0.9797$; $p = 0.3270$ 3-week VEH vs 3-week PNA $p > 0.9999$	Diff, -0.1663 [CI, -1.644, 1.312] $F_{(1,51)} = 0.05100$; $p = 0.8222$ 3-week PNA vs adult VEH $p = 0.9836$

Bold indicates $p < 0.05$.

In addition to the voltage-gated potassium currents mentioned above, voltage-gated sodium currents are critical for the initiation and propagation of action potentials, and also regulate neuronal excitability (Armstrong and Hille, 1998; Hille, 2001). In animal models of sensory neuropathies, mutations impairing fast inactivation of voltage-gated sodium channels increase excitability, hyperpolarize action potential threshold, and increase action potential FWHM (Xiao et al., 2019). In the present study, the lack of differences in these parameters, despite the differences in excitability, suggests that any changes in voltage-gated sodium channels in GnRH neurons from the groups studied are likely subtle. Voltage-gated calcium channels can also regulate neuronal excitability (Simons, 1988; Simms and Zamponi, 2014). Inhibition of T-type voltage-gated calcium channels in a subpopulation of medial habenula neurons reduces rebound burst firing and excitability (Vickstrom et al., 2020). Activation of voltage-gated calcium channel families can subsequently activate calcium-dependent potassium channels (Armstrong and Matteson, 1986; Storm, 1987), which can contribute to the shape and timing of the AHP and thus sculpt the firing activity of neurons (Sah and Faber, 2002). Calcium-dependent potassium currents have also been detected in GnRH neurons from mice and guinea pigs; these can

be modulated by estradiol feedback in mice, shape the AHP and contribute to both firing patterns and sub-threshold oscillations in these cells (Bosch et al., 2002; Liu and Herbison, 2008; Chu et al., 2012). Direct studies of specific currents will be needed to address these questions as changes to one or more voltage-gated currents cannot be ruled out by the present study, nor can PNA-induced changes despite the lack of effect of this treatment on overall excitability as quantified.

Development and PNA treatment could have independent and interacting effects on GnRH neuron activity and thus downstream reproductive function. The developmental increases in GnRH neuron excitability observed in the present study may help drive the pubertal reawakening of the reproductive neuroendocrine system. This may indicate intrinsic properties favoring firing become more important for effective neuroendocrine output in adults, perhaps because of concomitant changes in synaptic inputs. In PNA mice, the differences in spontaneous GnRH neuron firing (Dulka and Moenter, 2017) combined with the lack of PNA effects on stimulus-induced firing observed in this study focusing on overall intrinsic properties of these cells might indicate that it is primarily mechanisms upstream of GnRH neurons that are engaged by PNA treatment to increase spontaneous firing rate of

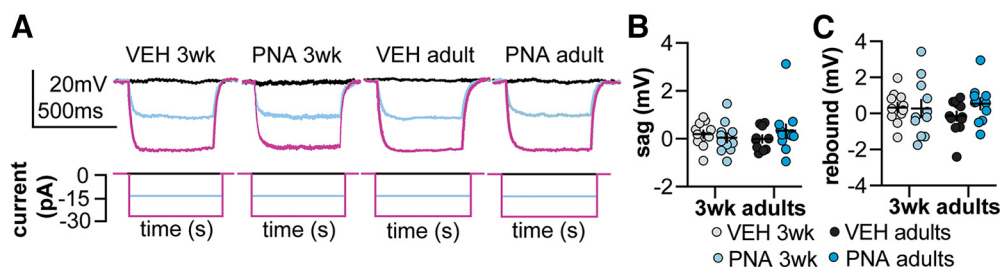


Figure 4. Neither development nor PNA treatment alter the response of GnRH neurons to hyperpolarizing current. **A**, Representative membrane voltage (top) responses to hyperpolarizing current injections (bottom); only three steps are shown for clarity. Individual values \pm SEM for sag (**B**), and GnRH neuron rebound following the hyperpolarizing current relative to baseline membrane potential (**C**). Statistical parameters shown in Table 5.

these cells in adults. In this regard, PNA treatment increases GABAergic neurotransmission to GnRH neurons at three weeks of age and in adulthood and increases appositions on GnRH neurons by GABAergic afferents originating in part from the arcuate hypothalamus (Moore et al., 2015). Increased activation of GABAergic afferents to GnRH neurons increases LH levels and LH pulse frequency (Silva et al., 2019). While LH is not a direct measure of GnRH neuron activity, pulse frequency is a good bioassay under most circumstances (Moenter, 2015). Together, these observations suggest increased GABAergic input to GnRH neurons correlates with increased GnRH neuron activity.

Other intrinsic mechanisms could contribute to altered response to GABA. First, it is possible that PNA treatment alters chloride cotransporter function. At the soma, there were no differences in the reversal potential of GABA_A-receptor-mediated current between control and PNA females at three weeks of age (Berg et al., 2018). It is not known, however, whether differences exist in the chloride homeostasis along GnRH neuron projections; if there is a lower intracellular chloride along the processes, for example, this could blunt the influence of more distal inputs. Second, PNA treatment may alter the number, type or sub-cellular location of GABA_A-receptors on GnRH neurons. One

Table 6: Mean \pm SEM and two-way ANOVA parameters for GnRH neurons hyperpolarized between -90 and -95 mV (Fig. 4)

Current (pA) needed to hyperpolarize the cell to −90 to −95 mV					
VEH 3 weeks	PNA 3 weeks	VEH adult		PNA adult	
−37.3 ± 1.95	−35.0 ± 1.78	−31.0 ± 2.67		−31.5 ± 1.73	
Steady-state to sag-peak membrane potential difference					
VEH 3 weeks	PNA 3 weeks	VEH adult		PNA adult	
0.2 ± 0.12	0.04 ± 0.20	−0.01 ± 0.15		0.3 ± 0.26	
Repolarization to basal membrane potential difference					
VEH 3 weeks	PNA 3 weeks	VEH adult		PNA adult	
0.3 ± 0.26	0.3 ± 0.48	−0.1 ± 0.26		0.5 ± 0.32	
Two-way ANOVA					
Property	Age	Treatment		Interaction	
Current (pA) needed to hyperpolarizethe cell to −90 to −95 mV	Diff, −4.867	Diff, −0.8671		Diff, −2.811	
	[CI, −8.960, −0.7745]	[CI, −4.960, 3.225]		[CI, −11.0, 21.915.374	
	$F_{(1,41)} = 5.768$; $p = 0.0209$	$F_{(1,41)} = 0.1831$; $p = 0.6710$		$F_{(1,41)} = 0.4811$; $p = 0.4918$	
	Bonferroni	3-week VEH vs 3-week PNA	3-week VEH vs adult VEH	3-week PNA vs adult PNA	Adult VEH vs adult PNA
		$p > 0.9999$	$p = 0.2398$	$p > 0.9999$	$p > 0.9999$
Steady-state to sag-peak potential difference	Diff, −0.04333	Diff, −0.09561		Diff, 0.4902	
	[CI, −0.4615, 0.3748]	[CI, −0.5138, 0.00928503226]		[CI, −0.3461, 1.327]	
	$F_{(1,41)} = 0.04380$; $p = 0.8353$	$F_{(1,41)} = 0.2132$; $p = 0.6467$		$F_{(1,41)} = 1.401$; $p = 0.2433$	
Repolarization to basal potential difference	Diff, 0.09892	Diff, −0.3164		Diff, 0.7403	
	[CI, −0.5909, 0.7887]	[CI, −1.006, 0.3735]		[CI, −0.6393, 2.120]	
	$F_{(1,41)} = 0.08386$; $p = 0.7736$	$F_{(1,41)} = 0.8579$; $p = 0.3597$		$F_{(1,41)} = 1.174$; $p = 0.2848$	

Bold indicates $p < 0.05$.

study in adults showed that PNA increases the amplitude of GABAergic postsynaptic currents and alters their kinetics in adulthood (Sullivan and Moenter, 2004), while another study did not show these alterations through development although a trend toward higher amplitude PSCs in adults was observed (Berg et al., 2018). It is important to bear in mind that these measures are made in the soma and that changes that occur in distal cell compartments may not be detected.

It is also possible that other afferents, including glutamate and neuropeptides, are altered by PNA treatment. In this regard, neurons in the hypothalamic arcuate nucleus called KNDy neurons use glutamate, kisspeptin, neurokinin B, and dynorphin to communicate. KNDy neurons are postulated to serve as a GnRH-pulse generator (Han et al., 2015). Of note, the frequency of LH pulses in female rodents and in both male and female sheep is increased by PNA treatment (Veiga-Lopez et al., 2008; Recabarren et al., 2012; Yan et al., 2014; Silva et al., 2019). KNDy neurons express receptors for gonadal steroids (Ruka et al., 2016; Silva et al., 2019), and thus serve as an important site for steroidal feedback modulation of GnRH neuron activity and a possible site of androgen action for the PNA phenotype (Pielecka-Fortuna et al., 2011; Yeo et al., 2014; Adams et al., 2018; Nagae et al., 2021). KNDy neurons have been studied in PNA mice, and while both GABAergic and glutamatergic appositions to these cells are reduced in PNA mice (Moore et al., 2021), neither the spontaneous firing rate nor the burst firing patterns of KNDy neurons change with development or with PNA treatment (Gibson et al., 2021). KNDy-mediated changes in input to GnRH neurons could be generated by alterations in neuromodulator expression (Goodman et al., 2013; Ahn et al., 2015). How other afferents to GnRH neurons change throughout development and/or with PNA treatment and whether they shape GnRH neuron firing remains to be studied.

Here, we demonstrate that changes to overall GnRH neuron excitability and action potential properties are primarily driven by age, and not altered by prenatal androgen exposure. Future work will focus on how specific ion channels are altered and how these changes shape the response of GnRH neurons to the input they receive. These findings contribute to the overall knowledge of GnRH neurons, how their intrinsic properties are shaped during development and with PNA treatment and generate testable hypotheses as to the cause of these developmental changes.

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