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Pubertal development of estradiol-induced hypothalamic progesterone synthesis

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ABSTRACT

In females, a hallmark of puberty is the luteinizing hormone (LH) surge that triggers ovulation. Puberty initiates estrogen positive feedback onto hypothalamic circuits, which underlie the stimulation of gonadotropin releasing hormone (GnRH) neurons. In reproductively mature female rodents, both estradiol (E2) and progesterone (P4) signaling are necessary to stimulate the surge release of GnRH and LH. Estradiol membrane-initiated signaling facilitates progesterone (neuroP) synthesis in hypothalamic astrocytes, which act on E2-induced progesterone receptors (PGR) to stimulate kisspeptin release, thereby activating GnRH release. How the brain changes during puberty to allow estrogen positive feedback remains unknown. In the current study, we hypothesized that a critical step in estrogen positive feedback was the ability for estradiol-induced neuroP synthesis. To test this idea, hypothalamic neuroP levels were measured in groups of prepubertal, pubertal and young adult female Long Evans rats. Steroids were measured with liquid chromatography tandem mass spectrometry (LC-MS/MS). Hypothalamic neuroP increases from pre-puberty to young adulthood in both gonad-intact females and ovariectomized rats treated with E2. The pubertal development of hypothalamic E2-facilitated progesterone synthesis appears to be one of the neural switches facilitating reproductive maturation.

1. Introduction

Steroid synthesis occurs throughout the nervous system. The ability of the brain to synthesize sex steroids from cholesterol was discovered over 35 years ago (Corpechot et al., 1985), but the function of neurosteroids remained elusive for many years. Many different neural cell types are capable of steroid synthesis, as evidenced by the presence of cholesterol transport proteins and steroidogenic enzymes, but the most steroidogenic cells in the brain are astrocytes (Zwain and Yen, 1999). Testosterone, estradiol, and progesterone are all locally synthesized de novo throughout various brain regions (for reviews see (Robel and Baulieu, 1994; Diotel et al., 2018)). Equally significant is that neurosteroids rapidly alter cellular signaling and even transcription through actions at the cell membrane. This membrane-initiated signaling modulates a variety of physiological and behavioral outcomes, including reproduction (reviewed in (Micevych et al., 2017)).

One of the most important neuroendocrine events associated with reproduction, the surge of luteinizing hormone that causes ovulation, requires hypothalamic synthesis of progesterone (Mittelman-Smith et al., 2017). Just prior to the LH surge, an estrogen-induced increase of hypothalamic progesterone (neuroP) occurs that is required for the LH surge (Micevych et al., 2003) and estrous cyclicity (Micevych et al., 2008). The E2 facilitation of hypothalamic neuroP synthesis occurs only in females, whereas E2 does not stimulate hypothalamic neuroP synthesis in males (Kuo et al., 2010). Stimulating primary astrocyte cultures from female and male rodents mirrors this sex difference: only hypothalamic astrocytes harvested from adult females exhibited an E2-facilitated increase in neuroP while male astrocytes were not responsive.

Puberty remains a mystery in spite of a great deal of work that has demonstrated that the brain undergoes profound changes that underlie the maturation of the reproductive system (Lomniczi and Ojeda, 2016). The maturation of GnRH signaling has been examined during puberty, including the expression of kisspeptin, a key excitatory signal to GnRH neurons. Kisspeptin expression increases across pubertal development in the anteroventral periventricular nucleus (AVPV) (Clarkson and Herbison, 2006; Mayer et al., 2010), a region within the rostral periventricular region of the third ventricle (RP3V), which integrates steroid information from the periphery (i.e. ovaries) as well as hypothalamic neuroP. One hypothesis holds that E2 and neuroP allow

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kisspeptin levels to reach a threshold during puberty, at which point there is sufficient activation of GnRH neurons to produce an estrogen positive feedback response – the hallmark of sexual maturation (Mittelman-Smith et al., 2015). In adulthood, estrogen positive feedback requires E2 from the ovaries and hypothalamic neuroP to stimulate kisspeptin neurons. When progesterone receptor is selectively knocked down in kisspeptin neurons, the LH surge is compromised, indicating that kisspeptin signaling requires the actions of neuroP for estrogen positive feedback. (Stephens et al., 2015), Interestingly, during prepubertal development, circulating levels of E2 spike several times, but do not induce puberty until the 3rd - 4th weeks of life, suggesting that rising peripheral levels of E2 are not sufficient to induce puberty. We hypothesize that hypothalamic synthesis of neuroP is low and not inducible by E2 pre-pubertally. We and others have demonstrated the importance of neuroP for kisspeptin expression both in vitro and in vivo (Mittelman-Smith et al., 2015; Marraudino et al., 2018), but whether neuroP increases during puberty to initiate kisspeptin signaling and puberty remains unknown.

In the present study, we examined neuroP levels in the rat hypothalamus before, during, and after pubertal development to determine whether hypothalamic neuroP increases during puberty. To confirm that age-related changes in neuroP were not solely due to circulating gonadal hormone levels, progesterone was measured in the hypothalamus of prepubertal, pubertal, and young adult gonadectomized rats given 50 μM estradiol benzoate, a dose used to stimulate an LH surge. The increase in hypothalamic neuroP across pubertal development is associated with maturation of reproductive circuits in female rodents.

2. Materials and methods

2.1. Animals

Female Long Evans rats were ordered from Charles River laboratories (Wilmington, MA) to arrive at three ages (on arrival): postnatal day (PND) 7 (shipped with dam), PND 26, and PND 48 ($n = 6$/experiment/age). Rats were given a week to acclimate after shipping and housed in a temperature and humidity controlled vivarium on a standard 12:12 light:dark cycle (with lights on at 0600 h). All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the University of California, Los Angeles Institutional Animal Care and Use Committee.

2.2. Experimental timeline

Experiment 1: To determine if hypothalamic progesterone levels are altered by pubertal development in naturally cycling female rats, hypothalami were collected from prepubertal, pubertal, and young adult female rats. Starting on PND 30, pubertal rats were monitored daily to determine the day of vaginal opening, which occurred between PND 32–34. Starting on PND 53, estrous cyclicity was monitored daily in adult rats using the Rat Vaginal Estrous Cycle Monitor (Stoelting, #59160). From PND 58 onward, daily vaginal smears were also taken to confirm estrous cycle stage (e.g. Goldman et al., 2007). Prepubertal and pubertal brains were collected at 1400 h on PND 17 and 35, respectively, and adult female brains were collected on the afternoon of proestrus (between PND 59–61; 1400 h).

Experiment 2: To determine if estradiol treatment is sufficient to induce hypothalamic neuroP synthesis at different points throughout pubertal development, PND 14 (prepubertal), PND 32 (pubertal), and PND 56 (adult), rats were gonadectomized under isoflurane anesthesia (bilateral incisions) four days prior to tissue collection. The following day, rats received subcutaneous injections of estradiol benzoate (EB; Sigma, E8515; 50 μg in safflower oil). Approximately 50 h after the EB injection, brains were collected.

2.3. Tissue collection

Rats were anesthetized with isoflurane and rapidly (within 3 min) decapitated; brains were removed and chilled. The hypothalamus was dissected with the following boundaries: rostral extent of the optic chiasm, rostral extent of the mammillary bodies, lateral edges of the tuber cinereum and the top of the third ventricle. Hypothalami were weighed, homogenized in 1 mL of phosphate buffered saline (Fisher Scientific, SH3025601), and frozen until analysis of neuroP.

2.4. Steroid extraction and measurement

2.4.1. Homogenization and extraction

Steroid extraction was similar to (Tobiansky et al., 2018) with some modifications. Steroids were extracted from 50 μL hypothalamus homogenate in PBS (mean tissue weight = 3.20 ± 0.09 mg). Deuterated progesterone internal standards were added to each sample to track recovery. After centrifugation, 1 mL of the supernatant was transferred and HPLC-grade hexane (500 μL) was added. After vortexing, the bottom layer was dried in a vacuum centrifuge (Thermo-Electron SPD111V) at 60 °C for 45 min and stored at −20 °C until resuspension. The calibration curve and blanks were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (calibration curve range: 0.4 to 1000 pg/tube).

2.4.2. LC-MS/MS measurement of steroids

Samples were transferred into a refrigerated autoinjector (15 °C) and 45 μL of the resuspended sample was injected into a Nexera X2 UPLC system (Shimadzu Corp; Kyoto, Japan). The sample was then passed through a KrudKatcher ULTRA HPLC In-Line Filter (Phenomenex; Torrance, CA) and Poroshell 120 HPH C18 guard column (2.1 mm; Agilent; Santa Clara, CA) before separation in a Poroshell 120 HPH C18 column (2.1 × 50 mm; 2.7 μm; Agilent) using 0.1 mM ammonium fluoride in MilliQ water for the mobile phase A (A), and HPLC-grade methanol for the mobile phase B (B) at a flow rate of 400 μL/min. The gradient was as follows: 0–0.5 min, 10% B; 0.6–4 min, 42% B; 9.4 min, 60% B; 9.5 min, 70% B; 11.9–13.4 min, 98% B; 13.5–14.9 min, 10% B. The needle was rinsed externally with 100% HPLC-grade isopropanol between each sample. Samples were detected with scheduled multiple reaction monitoring (sMRM), with two sMRM transitions for P4, and one sMRM transition for P4-d9. Data were acquired on a Sciex 6500 Qtrap triple quadrupole tandem mass spectrometer (Sciex LLC, Framingham, MA) in a positive electrospray ionization mode for P4.

2.5. Statistical analyses

Data are represented as means ± SEM. All statistical analyses were performed using GraphPad Prism 7. Separate one-way ANOVAs for gonadally intact (Exp 1) and OVX + EB (Exp 2) were performed to determine if there were statistically significant differences in neuroP levels at PND 15, 35, or 60. Significant main effects were followed up with Tukey's multiple comparisons tests. Statistical significance was considered if $p \leq 0.05$.

3. Results

3.1. Hypothalamic P4 synthesis increases from pre-puberty to young adulthood in female rats

Hypothalamic P4 levels differ across pubertal development in gonadally intact female rats (ANOVA, $F_{2,15} = 4.14$, $p = 0.037$; $n = 6$/grp; Fig. 1), such that there is a significant increase in hypothalamic P4 from pre-puberty/PND 17 (4.52 ± 0.79 ng/g) to young adulthood/PND 60 (21.1 ± 5.71 ng/g; $p = 0.032$), with peri-pubertal/PND 35

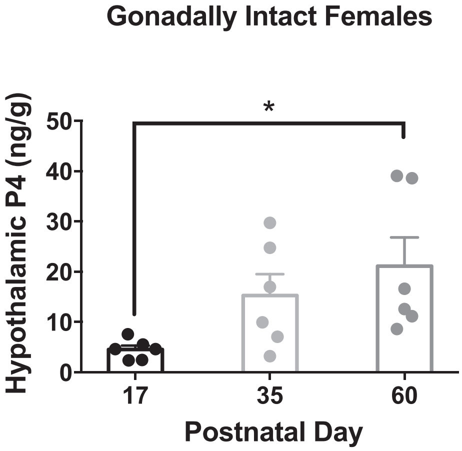

Fig. 1. Hypothalamic P4 increases across pubertal development in gonadally intact female rats. Hypothalamic P4 changes significantly over pubertal development (ANOVA, $F_{2,15} = 4.14$, $p = 0.037$) such that pre-pubertal (PND 17) females have less hypothalamic P4 compared with adult (PND 60) females in proestrus. $^*p < 0.05$.

intermediate to either age (15.28 ± 4.23 ng/g).

In gonadectomized and EB-treated rats, hypothalamic P4 levels also differ depending on stage of pubertal development (ANOVA, $F_{2,\ 14} = 4.49$, $p = 0.031$; $n = 5$–6/grp; Fig. 2), where there is a significant increase in hypothalamic P4 from puberty/PND 35 (2.3 ± 0.26 ng/g) to young adulthood/PND 60 (5.22 ± 0.99 ng/g; $p = 0.038$).

4. Discussion

The major finding of this study is that neuroP concentrations in the hypothalamus are developmentally regulated. Levels of neuroP steadily increase in the hypothalamus from prepuberty to adulthood, but E2-facilitated hypothalamic neuroP synthesis was seen only in post-pubertal female rats whether the female rats were gonadally intact or ovariectomized and EB-treated. The ability of only the post-pubertal female hypothalamus to respond to E2 by increasing neuroP is yet another indication of neural tissue changes during puberty that contribute to the maturation of reproductive circuits.

It has been challenging to accurately and precisely measure steroids in the brain using traditional immunoassay techniques, including radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA), in part because of the cross-reactivity of antibodies (Stanczyk et al., 2003; Holst et al., 2004). Liquid chromatography tandem mass spectrometry (LC-MS/MS) methods to measure steroids are more sensitive and specific compared with immunoassay methods (Monaghan et al., 2014; Koren et al., 2012; Stanczyk et al., 2007; Taylor et al., 2015; Taves et al., 2011). In some cases, immunoassays overestimate progesterone levels when compared with LC-MS/MS (Shankara-Narayana et al., 2016). In the current study, the use of LC-MS/MS to measure progesterone in small amounts of hypothalamic tissue (~3 mg) avoided antibody cross-reactivity with other progestins that might occur with traditional immunoassay methods. Specificity was assured by monitoring a quantifier ion transition and a qualifier ion transition, as well as a deuterated internal standard.

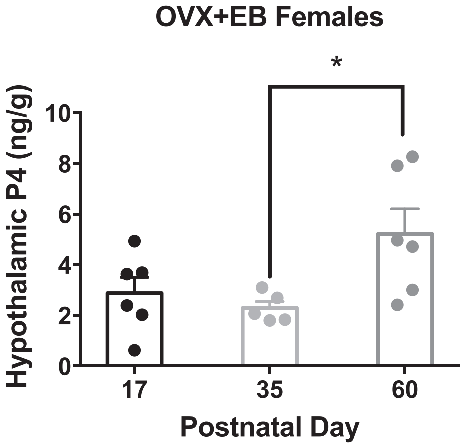

Fig. 2. Hypothalamic P4 increases during pubertal development in female rats that are ovariectomized (OVX) and given EB. Hypothalamic P4 synthesis changes significantly across pubertal development (ANOVA, $F_{2,\ 14} = 4.49$, $p = 0.031$) with a significant increase from puberty (PND 35) to adulthood (PND 60) in female rats that are OVX, given 50 μg EB, and tissue collected 50 h later. $^*p < 0.05$.

Levels of hypothalamic neuroP were very low in prepubertal rats and we could not detect any increase in neuroP synthesis after EB treatment. These low levels may represent a missing mechanism in the prepubertal hypothalamus that prevents E2 responsivity. Beginning at puberty, the E2-induced increase in neuroP may complete the maturation of reproductive circuits that allow for full stimulation of kisspeptin release onto GnRH neurons, causing the LH surge and ovulation. This model of neuroP action is based on both in vitro and in vivo experiments demonstrating that induction and release of kisspeptin requires neuroP and classic progesterone receptors (Mittelman-Smith et al., 2015; Mittelman-Smith et al., 2018; Stephens et al., 2015).

Because pubertal neuroP levels were intermediate to prepubertal and adult levels, it seems unlikely that neuroP is responsible for the *onset* of puberty, but rather may coordinate the timing and magnitude of the LH surge. Regular estrous cyclicity and ovulation begins 1–2 weeks after pubertal onset (Goldman et al., 2007), so the pubertal rats in this study most likely would not show regular estrous cyclicity or the ability to ovulate for 1–2 more weeks. We speculate that this is due to the need for a critical concentration of neuroP in the hypothalamus. Once the hypothalamus reaches a critical threshold/concentration of neuroP, we predict regular estrous cyclicity will ensue. The lack of neuroP increase in experiment 2 may have been the result of sampling. Previously, hypothalamic neuroP was dramatically elevated 45 h post-EB treatment (Micevych et al., 2003; Soma et al., 2005). In the present study, tissue was collected 50 h post-EB treatment, which was likely after the pre-ovulatory rise of P4. This idea is supported by the low levels of neuroP in hypothalamic tissue compared with intact females (~4 vs 20 ng/g tissue), suggesting that the pre-ovulatory peak of neuroP synthesis in the hypothalamus may have preceded tissue collection. Despite this, a clear developmental increase in neuroP was still observed and may be indicative of a more sustained surge of neuroP in adult females.

Astrocytes are the source of hypothalamic neuroP (Micevych et al., 2008; Kuo et al., 2009; Kuo et al., 2010). The observed developmental increase in neuroP synthesis in the hypothalamus between 5 and 8 weeks of age coincides with a pronounced astrocyte proliferation in the area of the brain that controls E2 positive feedback, the anteroventral periventricular nucleus (AVPV) (Mohr et al., 2016). It is tempting to speculate that these newborn astrocytes respond to ovarian E2 and are the source of neuroP in the adult hypothalamus. Indeed, inhibiting cell proliferation during puberty or young adulthood in female rats blunts and delays an induced LH surge (Mohr et al., 2017), suggesting that these newborn cells are needed to regulate the surge. Thus, pubertal development of reproductive competence is not only due to changes in cellular signaling in existing cells, but due to the addition of new astrocytes necessary for estrogen positive feedback. Implicit in this hypothesis is that the birth of estrogen-responsive astrocytes during puberty completes the maturation of the hypothalamus.

At this point we do not know whether there is regional specificity of E2-sensitive, neuroP synthesizing hypothalamic astrocytes. What is clear is that kisspeptin cells from the rostral periventricular nucleus of the third ventricle (RP3V), which includes the AVPV, integrate estradiol and progesterone signaling to stimulate GnRH neurons and initiate the LH surge (Herbison, 2008; Mayer et al., 2010; Kauffman et al., 2007). Astrocyte-derived neuroP augments E2-induced kisspeptin expression and release in immortalized kisspeptin cells that contain ERα and PGR and are characteristic of adult, female anterior hypothalamic kisspeptin neurons (Clarkson et al., 2008; Mayer et al., 2010; Poling et al., 2017; Mittelman-Smith et al., 2015, 2018). Moreover, PGR knockdown in kisspeptin neurons eliminates the LH surge (Stephens et al., 2015). Taken together, it is likely that astrocytes located near the RP3V are the source of hypothalamic neuroP needed for full stimulation of kisspeptin neurons.

What triggers puberty remains a mystery. While there are probably many events that are necessary in the brain for the reproductive circuitry to mature, the present studies demonstrate that an important component of this development is the ability of hypothalamic astrocytes to respond to E2 by increasing neuroP synthesis – a step that is needed for estrogen positive feedback. At this point it is not clear whether pre-existing astrocytes mature during puberty, or whether a population of new astrocytes is born during puberty that have the mechanism to respond to E2 with facilitated neuroP synthesis.

5. Conclusions

The current study demonstrates that hypothalamic E2-facilitated neuroP synthesis arises during puberty. These elevated levels of neuroP may be critical to ensure full stimulation of kisspeptin release, regular estrous cyclicity, and the LH surge leading to ovulation.

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