The Emerging Role of Chromatin Remodeling Factors in Female Pubertal Development

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Abstract

To attain sexual competence, all mammalian species go through puberty, a maturational period during which body growth and development of secondary sexual characteristics occur. Puberty begins when the diurnal pulsatile gonadotropin-releasing hormone (GnRH) release from the hypothalamus increases for a prolonged period of time, driving the adenohypophysis to increase the pulsatile release of luteinizing hormone with diurnal periodicity. Increased pubertal GnRH secretion does not appear to be driven by inherent changes in GnRH neuronal activity; rather, it is induced by changes in transsynaptic and glial inputs to GnRH neurons. We now know that these changes involve a reduction in inhibitory transsynaptic inputs combined with increased transsynaptic and glial excitatory inputs to the GnRH neuronal network. Although the pubertal process is known to have a strong genetic component, during the last several years, epigenetics has been implicated as a significant regulatory mechanism through which GnRH release is first repressed before puberty and is involved later on during the increase

in GnRH secretion that brings about the pubertal process. According to this concept, a central target of epigenetic regulation is the transcriptional machinery of neurons implicated in stimulating GnRH release. Here, we will briefly review the hormonal changes associated with the advent of female puberty and the role that excitatory transsynaptic inputs have in this process. In addition, we will examine the 3 major groups of epigenetic modifying enzymes expressed in the neuroendocrine hypothalamus, which was recently shown to be involved in pubertal development and progression.

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Introduction

The first endocrine indication of the initiation of puberty is an increase in pulsatile luteinizing hormone (LH) release [1, 2]. In girls, plasmatic LH pulse frequency increases after the initiation of sleep during the early puberty phase, even before any somatic manifestation becomes evident and given the lack of changes in circulating ovarian steroids. In rodents, increased LH pulse amplitude is also ovary independent and occurs in the afternoon by the end of the juvenile period [3, 4]. Elevated

plasma LH levels increase gonadal sex steroid production, leading to the development of secondary sexual characteristics. In females, upon maturation of the central mechanism of estrogen-positive feedback, the first pre-ovulatory surge of gonadotropins occurs followed by ovulation.

The pubertal change in LH output is driven by an increased pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus [5]. Because GnRH neurons are capable of producing and releasing GnRH long before puberty, the pubertal changes in GnRH activity are primarily due to changes in the transsynaptic balance of activating versus repressing neuronal and glial inputs to GnRH neurons [5]. The initiation of puberty has been hypothesized to result from the removal of a central “brake” [6]. According to this hypothesis, the secretory activity of GnRH neurons is predominantly under transsynaptic inhibitory control during the infantile period. There are some species differences: while the inhibitory transsynaptic control of GnRH neurons is primarily provided by GABA neurons in primates and rodents [7, 8], NPY neurons are inhibitory in primates [9] and excitatory in rodents [10–15]. The strength of the opioid inhibitory tone diminishes at the time of puberty in rodents [16–20], and RFamide-related peptide-containing neurons seem to be involved in the inhibition of mouse GnRH neuron pulse frequency during infancy [21], pubertal delay induced by dexamethasone [22], or advancement induced by bisphenol A [23]. Overall, when central inhibition is lifted at puberty, there are increased excitatory inputs to the GnRH network that result in increased GnRH release [5, 24].

Robust excitatory inputs to GnRH release are provided by glutamatergic neurons [25, 26] as well as a family of peptides known as kisspeptins [27–30]. All kisspeptins are derived from the same kisspeptin precursor that is the product of the *KISS1/Kiss1* gene [31, 32]. Kisspeptins are vital for puberty to occur, since mutations in *Kiss1* and inactivating mutations in its cognate receptor (*Kiss1r/GPR54*) result in infertility and a lack of puberty [33, 34]. Lastly, glial cells contribute to the hypothalamic control of puberty [35, 36] by releasing small molecules (e.g., ATP, E2, and PGE2) and growth factors, as well as through cell-cell interactions that require direct glia-GnRH neuron contact [35, 37, 38], enhancing GnRH release (Fig. 1).

The primary transsynaptic mechanism controlling pulsatile GnRH release is thought to be the coordinated activity of a subset of neurons of the arcuate nucleus (ARC) called KNDy neurons [39, 40], so named because they produce kisspeptin, NKB, and dynorphin [39, 41].

KNDy neurons release NKB, which acts upon other KNDy cells, ultimately stimulating kisspeptin release [39, 41]. NKB and kisspeptin are released in a rhythmic manner that is primarily determined by the inhibitory effect of dynorphin on NKB release [39, 41]. Direct evidence for a role of KNDy neurons in pulsatile LH release was recently provided [42]. There is a second population of kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) of rodents [43]. These AVPV neurons are necessary for the preovulatory surge of gonadotropins [43] and also innervate the ARC in mice [44], suggesting a possible role of AVPV kisspeptin neurons in the onset of puberty.

Despite the importance of this transsynaptic regulatory mechanism, recent evidence suggests the existence of a molecular mechanism that controls the timing of puberty by epigenetically regulating the transcriptional activity of neurons involved in stimulating GnRH release [45–48]. There are no studies describing the epigenetic changes in the inhibitory transsynaptic control mechanism of the juvenile to pubertal transition. This review will focus mostly on the epigenetic control of kisspeptin neurons (mainly KNDy neurons of the ARC) during the juvenile to pubertal transition.

Epigenetic Regulation of Gene Expression

To allow the long chains of DNA to be packed into eukaryotic nuclei, cells wrap DNA around histone octamers in a structure called chromatin. When this structure is condensed (aka heterochromatin), it is mostly inactive or transcriptionally repressed. In contrast, in the “open” state (aka euchromatin), the transcriptional machinery has free access to DNA for gene expression. The regulation of chromatin states is controlled by a variety of chromatin-modifying enzymes that modify both histones or DNA itself. These epigenetic modifications induce chromatin remodeling that alters gene expression and ultimately impacts cellular phenotypes. DNA methylation is the most commonly studied epigenetic modification, whereas the greatest diversity of modifications occurs on histones, the tails of which can undergo methylation, acetylation, ubiquitination, phosphorylation, sumoylation, crotonylation, deimination, butyrylation, ADP ribosylation, propionylation, formylation, hydroxylation, and O-GlcNAcylation.

Epigenetic modifying enzymes can be divided into 3 broad categories: *epigenetic writers* add epigenetic marks onto DNA or histones that are removed by *epigenetic*

Fig. 1. Hypothalamic regulation of pulsatile GnRH release. The control of puberty is exerted by excitatory and inhibitory trans-synaptic inputs to GnRH neurons. The current concept claims that the initiation of puberty implicates a shift from a mainly inhibitory to a stimulatory mode of control. This results in the diurnal activation of pulsatile GnRH release, leading to increased LH pulsatility, which is the first endocrine manifestation of puberty. Changes in pulsatile GnRH release are due to decreased inhibitory

neurotransmission (GABA, opioids, RFamide) and concomitant increased activation of stimulatory neuronal (glutamate, kisspeptins) and glial (TGF α , PGE2) networks that operate within the ARC of the hypothalamus. These neuronal and glial systems affect GnRH pulsatility by predominantly targeting GnRH nerve terminals reaching the median eminence. The preovulatory surge of gonadotropins is triggered by the positive feedback of estradiol on AVPV kisspeptin neuronal activation.

erasers, while *epigenetic readers* recognize these marks and recruit other modifying enzymes and transcription factors. This review will focus on the epigenetic modifying enzymes/processes that have been shown to impact the neuroendocrine control of pubertal development (Fig. 2).

DNA Methyltransferases as Epigenetic Writers

The most studied epigenetic modification of DNA in mammalian cells is the covalent addition of a methyl group to the C5 position of the cytosine of the dinucleotide sequence CpG [49, 50] by DNA methyltransferases (DNMTs), resulting in the formation of 5-methylcytosine (5-mC). The presence of 5-mC in gene promoter regions promotes the recruitment of methyl-binding proteins that ultimately induce heterochromatin formation and gene silencing [51]. Oxidation of 5-mC by the TET family of dioxygenase enzymes produces 5-hydroxymethylcytosine (5-hmC) [52, 53], an epigenetic modification linked with active gene transcription [54, 55]. The balance between these posttranslational modifications

(PTMs) depends on the enzymatic activities of DNMTs and TET. Although DNMT1 functions in maintaining basal DNA methylation, DNMT3a and DNMT3b are required for de novo methylation [49].

Gonadal steroids impact neuronal sexual differentiation in the neonatal rodent brain, generating differences that later influence adult reproductive physiology and behavior. The preoptic area, which is involved in sexual and maternal behavior, is highly regulated by estradiol. DNA methylation of the estrogen receptor alpha (ER α) gene has been shown to influence the estrogen-dependent sexual differentiation of the preoptic area in rodents [56] as determined between birth and 3 weeks of life in the mouse. Although some portions of the ER α promoter show a DNA methylation pattern that does not correlate with gene expression [57], other portions of the promoter exhibit DNA methylation with good correlation to gene expression [58], suggesting that the overall changes are confined and limited to specific CpGs in portions of the 5' flanking region of the ER α promoter.

The expression of the *Kiss1* gene in the AVPV area is sexually dimorphic [59]. While AVPV *Kiss1* expression is higher in adult females than in males [60], the level of CpG

Fig. 2. Chromatin structure and the epigenetic code. Heterochromatin is a tightly packed combination of DNA and histone octamers that is associated with less active gene transcription. Euchromatin is less condensed and associated with active gene transcription. Epigenetic writers (brown hexagons) modify the amino acid residues of histone tails, building the histone code at DNA regulatory regions. Epigenetic readers (blue semi-circles) bind to these epigenetic marks and help recruit transcription factors and other epigenetic modifiers. Epigenetic erasers (yellow circles) catalyze the removal of epigenetic marks. The balance of these enzymatic pathways, and thus the balance between hetero- and euchromatin, is what renders a gene in active or silent state.

methylation at the promoter region is higher in females than in males [60]. Although counterintuitive, it has been shown that if DNA methylation is associated with silencer regulatory regions, then the recruitment of methyl-binding proteins and the formation of heterochromatin block the recruitment of transcriptional repressors, leading to an up-regulation of gene expression [51, 60].

Comparative studies of DNA methylation using whole-genome bisulfite sequencing with RNA sequencing (RNA-seq) demonstrated that changes in DNA methylation at promoter regions, as well as introns and untranslated regions, influence gene expression in the goat hypothalamus during the onset of puberty [61]. Furthermore, studies per-

formed in nonhuman primate cultured GnRH neurons show that there is a decrease in methylation throughout development at multiple CpG sites upstream of the *GNRH* transcription starting site, indicating that the loss of methylation of regulatory regions of the *GNRH* gene eliminates its repression, facilitating *GNRH* transcription [62]. As a result of this deregulation, *GNRH* mRNA expression increases between 14 and 18 days in in vitro cultures, reaching a maximum peptide release that agrees with the observed decrease in DNA methylation [62].

The existence of a pubertal brake is now a widely accepted concept [6]. During the prepubertal period, the secretory activity of GnRH neurons is under transsynap-

Fig. 3. Diagram depicting the changes in histone marks and epigenetic enzymes at the promoter region of the *Kiss1* gene during the infantile to pubertal transition. During the infantile period, when LH pulsatility is low, the expression of several epigenetic repressors (*Eed*, *GATAD1*, and *SIRT1*) is elevated in the ARC. The presence of these epigenetic repressors at the *Kiss1* promoter/enhancer region produces a histone landscape associated with transcriptional repression (high levels of H3K27me3 and low levels of H3K4me3, H3K9/14ac, and H4K16ac). As a result of this, *Kiss1*

expression remains low. As puberty progresses, the expression of these epigenetic repressors diminishes, while the expression of members of the TrxG of epigenetic activators increases. Increased binding of MLL1 and MLL3 to the *Kiss1* promoter and enhancer region, respectively, shifts the epigenetic landscape of the *Kiss1* regulatory region into a more active state (low levels of H3K27me3 and high levels of H3K4me3, H3K9/14ac, and H4K16ac). This results in increased recruitment of RNA polymerase 2 and enhanced *Kiss1* expression at puberty.

tic inhibitory control. At the onset of puberty, this inhibition is lifted, resulting in the reactivation of GnRH release [63]. Since the *Kiss1* gene is one of the primary puberty activators whose expression increases during the infantile to pubertal transition, we hypothesized that during the infantile period, there should be a high expression of transcriptional repressors, and the expression of repressors should diminish as puberty unfolds, allowing for *Kiss1* mRNA levels to increase. In an attempt to identify mRNA expression and DNA methylation changes in the rat ARC during the infantile to pubertal transition, we observed reduced expression of 2 members of the Polycomb group (PcG) of epigenetic repressors, *Eed* and *Cbx7*, while methylation of their promoter regions increased [45]. Later, we determined that the PcG proteins maintain low ARC *Kiss1* expression during the infantile period, and as pu-

berly unfolds, PcG expression decreases, allowing for *Kiss1* expression to increase [45]. These results suggest that de novo methyltransferases, such as DNMT3a and DNMT3b, could be responsible for the reduction in PcG protein expression at puberty. More research is needed to identify the gene networks that are controlled by these enzymes and to determine how they are targeted to specific loci throughout the genome.

PcG Proteins as Epigenetic Writers

In mammals, PcG proteins form two primary repressive complexes (PRC1 and PRC2) [64, 65]. Gene silencing by PcG proteins begins with the recruitment of the PRC2 complex to Polycomb response elements (PREs) and the

induction of trimethylation of histone H3 at lysine 27 (H3K27me3), a mark associated with silent chromatin [66]. H3K27me3 is subsequently recognized by CBX members of the PRC1 complex that bind to this modification through their chromodomain, producing a robust repressive complex [64, 65, 67].

In an effort to understand the role of PcG transcriptional repressors during pubertal development and using the *Kiss1* gene as prototype, we showed that 2 important PcG members, *Eed* and *Cbx7*, are expressed in ARC kisspeptin neurons and associate with the *Kiss1* promoter during the prepubertal period [45]. Moreover, as puberty approaches, PcG association to the *Kiss1* promoter decreases, inducing a reorganization of the chromatin landscape associated with increased levels of H3K9ac, H3K14ac, and H3K4me3, all epigenetic marks associated with gene activation. Finally, as expected by these changes, *Kiss1* mRNA levels increase, allowing puberty to progress [45].

As the repressive writers of the PcG are removed from the *Kiss1* promoter region, the chromatin hyperacetylation observed at puberty could be due to increased histone acetyltransferase (HAT) and/or decreased histone deacetylase enzymatic activity. Similarly, the increased trimethylation of histone 3 at lysine 4 could also be due to the decreased activity of erasers, such as histone demethylases, and/or the increased activity of writers, such as histone methyltransferases. One of the candidates for this activity is the Trithorax group (TrxG) of epigenetic writers due to their recognized PcG-antagonizing activity [64, 68] (Fig. 3).

TrxG Proteins as Epigenetic Writers

The TrxG proteins are evolutionary conserved chromatin modifiers that are classified into 3 classes: the first class of proteins methylates histone tails; the second class contains ATP-dependent chromatin remodeling factors, including “readers” of histone methylation marks; and the third class binds to specific DNA regions and is comprised of histone modifiers, chromatin remodelers, and other unclassified proteins [69]. TrxG proteins counteract the repressive effect of PcG proteins by adding the activating H3K4me3 epigenetic mark at gene regulatory regions. The increased H3K4me3 mark is mediated by either the complex of proteins associated with Set1 (COMPASS) or by COMPASS-like complexes, named after the first methyltransferase, SET1, discovered in yeast [68, 69]. The protein complexes described to date contain

one of the following methyltransferases at their core: Su(var)3–9, enhancer of zeste, Trithorax (SET1)A or SET1B, mixed-lineage leukemia 1 (MLL1) or MLL2, and MLL3 or MLL4 [68]. Most importantly, while SET1A/SET1B COMPASS mediates the deposition of the H3K4me3 mark at the promoter regions of active genes [70], MLL1/MLL2 performs this function at promoters that are ready for activation or “bivalent” [71–73], while the MLL3/MLL4 complexes catalyze the activation of enhancer sites [70].

To understand the role of the TrxG complex as an opposing force to the PcG proteins in the increased *Kiss1* expression at the juvenile to pubertal transition, we used siRNA-mediated ARC-specific downregulation of *Mll1* mRNA expression. Decreased *Mll1* expression induced the loss of MLL1 and reduced H3K4me3 to the *Kiss1* promoter, with a concomitant inhibition of *Kiss1* expression and delayed puberty [47]. Additionally, using a CRISPR-CAS9-KRAB-mediated epigenetic remodeling approach to increase the inhibitory H3K9me3 histone mark at a novel *Kiss1* enhancer site, we inhibited the activity and binding of MLL3 on the *Kiss1* enhancer region and were able to block the pubertal rise in *Kiss1* expression and delay pubertal development [47]. These results demonstrate that by applying key histone PTMs, the TrxG is capable of altering the epigenetic landscape of the *Kiss1* promoter/enhancer region from a repressed to an active state at the time of female puberty (Fig. 3).

GATAD1/KDM1A Complex as an Epigenetic Reader/Eraser

Our lab has recently shown that some members of the zinc finger (ZNF) family of transcriptional repressors are important for keeping the GnRH pulse generator in check during prepubertal development by preventing the reawakening of GnRH secretion in nonhuman primates [46]. When *KISS1* and *TAC3* mRNA expression increases in the rhesus monkey hypothalamus during the juvenile-pubertal transition, *GATAD1* expression decreases. Additionally, when *GATAD1* is overexpressed in the ARC of immature rats, puberty is significantly delayed and the estrous cycle is disrupted [46]. GATAD1, a chromatin reader, recruits KDM1A, a histone eraser that induces the loss of activating H3K4me2/3 marks at gene promoter regions [74, 75]. Using an in vitro approach, we demonstrated that when GATAD1 is overexpressed, KDM1A recruitment increases to both the *KISS1* and *TAC3* promoters, inducing a significant reduction in the activating

H3K4me2/3 marks [46]. These results are in line with the idea that GATAD1 reduces *KISS1* and *TAC3* gene expression, partially by facilitating the loss of H3K4me2 marks from puberty-activating gene promoters via the gain of KDM1A. The decreased association of GATAD1 and KDM1A to the *KISS1* and *TAC3* regulatory regions and the concomitant increase in H3K4me2 abundance detected in the monkey medial basal hypothalamus at the time of puberty support the in vitro results, as well as the notion that there is a mechanism of epigenetic repression that is lost during the reawakening of the GnRH pulse generator throughout the juvenile-pubertal transition in the rhesus monkey hypothalamus [46] (Fig. 3).

Chromatin Remodeling Factors as Mediators of Environmental Signals

The epigenetic machinery plays an essential role in developmental programming [76–79], linking early-life changes in energy availability to diverse adult metabolic disorders [77, 79–81]. Since cellular metabolites are used as cofactors of histone PTMs [82], it is likely that alterations in the metabolic state that affect reproductive maturation would use epigenetic mechanisms to modify the expression of specific genes. The sirtuins, a class of histone deacetylases (HDACs) [78, 80, 83, 84], are candidates for assessing epigenetic links between nutritional input and the neuroendocrine control of puberty.

Because SIRT1 enzymatic activity depends on cellular nicotinamide adenine dinucleotide (NAD⁺) availability, SIRT1 acts as a fuel-sensing enzyme that can silence gene expression by promoting the loss of activating acetylated histone marks in response to nutrient availability [80, 83–86]. In a recent study, we demonstrated that when puberty is delayed under conditions of undernutrition, SIRT1 expression is upregulated in the ARC [48], as observed in other models [87–89]. The opposite result is observed in conditions of overnutrition, where SIRT1 is downregulated and puberty is advanced [48]. The epigenetic eraser SIRT1 affects ARC *Kiss1* promoter chromatin structure by deacetylating histone 4 at lysine 16 (H4K16ac) and histone 3 at lysine 9 (H3K9ac) [48, 80], inducing heterochromatin formation and the downregulation of *Kiss1* expression. In addition, SIRT1 participates in PcG protein recruitment to *Kiss1* regulatory regions [48], which has also been shown in other systems [90–94]. These results suggest that SIRT1 may serve as an epigenetic relay between energy balance, gene expression, and reproductive function.

Advanced Puberty and Mutations at the Makorin 3 Locus

Patients with heterozygous loss-of-function mutations of the makorin 3 (*MKRN3*) gene have been observed to exhibit precocious puberty of central origin [95–105]. *MKRN3* is located in the Prader-Willy syndrome region of chromosome 15q11.2, and because it is a maternally imprinted intronless gene, individuals only inherit the mutation from their fathers [95]. *MKRN3* is a zinc finger protein with a C3HC4 motif called a RING domain that is associated with E3 ubiquitin ligase activity [106]. Ubiquitin ligases transfer ubiquitin from an E2-ubiquitin-conjugating enzyme to target proteins. *Mkrn3* is highly expressed in the ARC of juvenile mice, and its expression decreases as puberty progresses, suggesting it has a repressing function on the GnRH network [95]. Makorins have been implicated in the regulation of RNA polymerase 2 activity, suggesting they could be involved in transcriptional and/or epigenetic modes of regulation [107]. In vitro promoter assays have revealed that *MKRN3* represses *Kiss1* and *Tac3* promoter activity [unpubl. data], giving rise to the tantalizing possibility that *MKRN3* functions as a transcriptional repressor or even as a repressive epigenetic writer via its ubiquitin ligase activity. Moreover, since both the *MKRN3* and *GATAD1* repressors show the same expression changes in the ARC, it is possible that both genes could be controlled by a common regulatory pathway or even form part of the same repressive complex. Additional research is needed to determine the role of *MKRN3* in the maturation of the hypothalamus and in the pathogenesis of central precocious puberty.

Future Perspectives

There has been an explosion in the development of new genomic and transcriptomic technologies in the last few years that has helped fuel novel research aimed at understanding the dynamic relationship between the environment, the cellular state, and the epigenome. Some of the new technologies that have emerged include the following. (1) Precision nuclear run-on sequencing (PRO-seq), a technique allowing the expression of nascent mRNAs, enhancer RNAs (eRNAs) and noncoding RNAs to be assessed by detecting changes in the endogenous polymerase activity compared to the expression of all the mRNAs present in a cell at any given time. This technique enables us to determine the precise timing of

polymerase activation and mRNA synthesis. (2) Assay for transposase-accessible chromatin using sequencing (ATAC-seq) takes advantage of open chromatin regions being easily attacked by the Tn5 transposase to produce single nucleotide resolution maps of open regulatory elements throughout the genome. This technique is used to identify regulatory regions (enhancer and promoters) associated with gene expression at the single-cell level. (3) Chromosome conformation capture assays are a large variety of assays designed to analyze the spatial organization of the chromatin in the nucleus. Chromosome capture enables the study of promoter-enhancer interactions and long-distance intra- and interchromosomal contacts. The use of these technologies will shed new light on how the cell translates external information into genome changes that have long-lasting effects throughout development.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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Author Contributions

C.F.A. and A.L. wrote the manuscript. K.V.-C. prepared the figures.

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