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Hypothalamic Epigenetics Driving Female Puberty

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Abstract

Puberty involves a series of morphological, physiological and behavioral changes during the last part of the juvenile period that culminates in the attainment of fertility. The activation of the pituitary-gonadal axis by increased hypothalamic secretion of Gonadotropin Releasing Hormone (GnRH) is an essential step in the process. The current hypothesis postulates that a loss of transsynaptic inhibition together with a rise in excitatory inputs are responsible for the activation of GnRH release. Similarly, a shift in the balance in the expression of puberty activating (PA) and puberty inhibitory (PI) genes exists during the pubertal transition. In addition, recent evidence suggests that the epigenetic machinery controls this genetic balance, giving rise to the tantalizing possibility that epigenetics serves as a relay of environmental signals known for many years to modulate pubertal development. Here we review the contribution of epigenetics as a regulatory mechanism in the hypothalamic control of female puberty.

Keywords

GnRH neurons; KNDy neurons; kisspeptin neurons; arcuate nucleus; female puberty; chromatin modifications; transcriptional repression; transcriptional activation; epigenetic regulators; Polycomb group; Trithorax complex

Introduction

Puberty is the process through which a child maturates into an adult capable of sexual reproduction. This process is accompanied by body growth as well as development of secondary sexual characteristics [1]. Form a purely neuroendocrine perspective, the onset of puberty requires the activation of hypothalamic secretory neurons that produce and release GnRH [2] in pulsatile fashion. Pulsatile GnRH release activity depends on excitatory and/or inhibitory transsynaptic and glial inputs originated from either neuronal subsets or glial cells interconnected to the GnRH neuronal network [2–5].

For a long time, studies have acknowledged that puberty has a strong genetic component and in recent years, epigenetics has been recognized as a key regulatory mechanism by which GnRH release is first kept in place before puberty, and later it becomes in charge of

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modulating the increased GnRH secretion in order to initiate the pubertal process. In this review, we will discuss the concept supporting epigenetics as a fundamental mechanism providing gene-specific gatekeeper functions [6] and the plasticity needed to temporary modify gene expression [7;8] during pubertal development. It is well-known that these mechanisms are essential to an adequate development and differentiation of distinct cell lineages in the organism. These processes can also be modified by exogenous influences, and as such, can contribute to or be result of the environmental alterations of the phenotype. Epigenetic programing has a decisive role in modulating pluripotency genes that become inactive during differentiation [9–11]. This machinery is also indispensable for a number of neural processes, including sexual differentiation of the brain [12;13], learning and memory formation [7;14], neuronal plasticity [15], dendritic development [16], and glial-neuronal interactions [17]. In addition, we will focus our discussion on the emerging evidence that supports the idea of a dynamic epigenetic machinery that regulates the neuroendocrine core process controlling the initiation of female puberty.

Neuroendocrine regulation of puberty: Neuronal circuits, GnRH pulsatility and LH release

In humans and non-human primates, during early postnatal life, Luteinizing Hormone (LH) pulsatility is high in a period called “mini puberty” [18;19]. In rodents, mini puberty is manifested by a transient increase in testosterone during early postnatal hours [20;21] in males. Whereas, in female rats and mice there is a transient GnRH dependent increase in Follicle-Stimulating Hormone (FSH) release around postnatal day 12, responsible for enhancing the development of preantral follicles as well as rescuing early antral follicles from apoptotic death [22–26]. This phase is followed by infancy, characterized by a marked quiescence of the gonadotropic axis. During the late juvenile period in rodents, or early pubertal period in humans and primates, there is an awakening of the GnRH pulse generator and therefore increased LH pulsatility [18;19].

It is well established that the first endocrine manifestation of puberty in humans is an increase in nocturnal pulsatile frequency of plasma LH, detected during the early stages of pubertal development [27]. Subsequent studies on other mammalian species have also evidenced an increase of pulsatile patterns of LH although during daytime, this is the case for nonhuman primates, rodents and sheep [2].

GnRH neurons are controlled by counteracting excitatory and inhibitory inputs that, within defined areas of the hypothalamus, regulate GnRH secretion. Strong excitatory inputs to GnRH release are provided by glutamatergic neurons [2;3] as well as a family of peptides known as kisspeptins [28–31]. These peptides are originated by a same kisspeptin precursor which are the product of the KiSS-1 Metastasis-Suppressor (*KISS1/Kiss1*) gene [32;33]. The presence and function of kisspeptins is vital for puberty to occur [34]. On the other hand, inhibitory transsynaptic regulation of GnRH neurons is mostly provided by gamma-aminobutyric acid (GABAergic) neurons [35;36], opiatergic neurons [35], and arginine and amidated phenylalanine (RFamide)-related peptide-containing neurons [37;38]. Finally, glial cells also collaborate to the hypothalamic control of puberty [4;5], where astrocytes and

tanycytes expedite GnRH secretion by i) releasing small molecules like adenosine triphosphate (ATP), estradiol (E2), prostaglandin E2 (PGE2), and growth factors, and ii) via cell-cell interactions which require direct glia-GnRH neuron contact [5;39;40].

The transsynaptic mechanisms underlying GnRH pulsatility proceeds from the coordinated activities of a set of neurons located in the Arcuate Nucleus (ARC) of the hypothalamus. These neurons are known as KNDy because they secrete kispeptin, neurokinin B (NKB) and dynorphin [41;42]. While NKB stimulates kisspeptin release from other KNDy neurons in a positive feedback loop, the phase shifted inhibitory action of dynorphin on KNDy neurons produces an oscillatory behavior [42;43].

Besides the ARC, other populations of kisspeptin neurons have been found in the rostral periventricular area of humans and rodents, and in the anteroventral periventricular nucleus (AVPV) of rodents [44–46]. Kisspeptin neurons in the ovine brain show a similar pattern of distribution [47]. These kisspeptin neurons do not produce NKB or dynorphin and they do not contribute to the modulation of GnRH pulsatility, but they are necessary for the preovulatory surge of gonadotropins after the pubertal process has started [48].

The initiation of puberty takes place after the removal of a central restraint that keeps GnRH release in check [49–52]. Up to this point, during the prepubertal or infantile period, the secretory activity of GnRH neurons is under a predominant transsynaptic inhibitory control. Once puberty is underway, the inhibition lock is lifted, there is a concomitant increase in excitatory inputs to the GnRH network provided in part by activated kisspeptin neurons [48;53], ultimately resulting in increased GnRH release. Overall, it is now accepted that a joint decrease in inhibitory inputs together with an increase in excitatory neurotransmission are essential antagonistic mechanisms through which the pubertal process gets initiated [54–65]. Recent evidence shows that this inter-cellular mechanism of excitatory vs. inhibitory balance is mirrored at the genomic level. Three gene groups have emerged: as either PI genes (e.g. *GAD67* [35;36], *PDYN* [35], *GnIH* [37;38]), PA genes (e.g. *GnRH* [66;67], *GLS* [2;3], *KISS1* [32;33], *TTF1* [68;69]) or genes with dual effects depending on hormonal milieu and cell identity (e.g. *TAC3* [42;70–72], *EAP1* [73;74]).

Decoding pubertal gene networks

The well-organized output of biological signals that influence the time of puberty are heavily affected by a variety of genes that work in coordinated networks operating in the hypothalamus. The Tumor Related Genes (TRG) is a good example of a network of genes operating within the hypothalamus, that regardless of bearing a wide-range of cellular functions, are all related to tumor suppression or tumor formation [75]. In silico studies in rats and non-human primates have suggested that TRGs show a well-defined network structure with central node members, named hubs, which are the core components, and a number of subordinated genes placed peripherally that are transcriptionally regulated by the central nodes. The five putative central nodes of the TRG are strongly connected between them and to other upper-echelon TRG genes (*EAP1*, *TTF1* and *OCT2*) suggested also to be implicated in the transcriptional regulation of the pubertal process [68;73;76]. Experimental validation has been demonstrated for the upper-echelon gene *EAP1* [77], which is part of a

repressive complex that modulates apoptosis in breast cancer [78], and for the subordinate gene *KISS1* [79] previously known as a metastasis suppressor gene [80]. *TSLC1* (also known as *SynCAM1*) has an important role in glia-GnRH neuron adhesive communication during development, and also in astrocytic facilitation of GnRH secretion towards a correct reproductive function [81;82].

Other transcriptional gene networks related to puberty have been described in cattle [83]. Network connectivity analysis of core components have shown similar TRG-related target genes like *NELL2* and *NRG1*, the latter encoding for an astrocytic *erbB4* receptor ligand. The Trithorax group (TrxG) member Mll3 was identified as part of the top 10% most connected transcription factors during bovine puberty [83]. *Mll3*, expressed in the bovine hypothalamus, serves as a central hub by regulating the expression of 39 target genes [83].

As new gene interactions are unveiled, new network models become available and modified according to the current information. For example, a Genome Wide Association Study (GWAS) performed on a cohort of Japanese population discovered a Single-Nucleotide Polymorphism (SNP) associated to precocious menarche. This SNP was near the *TTF1* locus, known as *Nkx2.1* [84]. Moreover, network connectivity analysis showed that *TTF1* and *EAP1* serve as central hubs to a number of genes associated to menarche [85–87] including: *GAB2*, *NR4A2*, *ZNF462*, *ZNF483*, *TMEM18*, *PAX8*, *KDM3B*, *KISS1* and *KISS1R* (for an extended gene list see [69]). These menarche-related genes are connected to two or more TRG central nodes as determined by GeneMANIA network software [88] and available databases. Genes connected to more than one TRG become potential regulators of gene expression. This is the case of lysine demethylase KDM3B, which is implicated in the removal of repressive histone marks, monomethylation and dimethylation of histone 3 at lysine 9 (H3K9me1/me2) [89;90]. Overall, these studies show the existence of strong interrelationships in expression between TRG central nodes, puberty genes, and menarche-related genes discovered using different platforms. Notwithstanding the possible role of these gene regulatory networks on pubertal development, evidence is accumulating suggesting an epigenetic layer of regulation of the neurons involved in stimulating GnRH release.

Mechanisms of epigenetic control

Epigenetics refers to hereditable modifications that are not due to changes in the DNA sequence. These alterations ultimately modify the DNA accessibility and chromatin structure, thus controlling different patterns of gene expression. There are three well-known mechanisms of epigenetic regulation: First, by chemical alterations of the DNA via DNA methylation and hydroxymethylation. Second, by modifications of the chromatin structure caused by posttranslational modifications (PTMs) of histones, which carry the protein component of the nucleosome, the core unit of chromatin. The third mechanism of epigenetic control is through non-coding RNAs (ncRNAs), providing epigenetic information as either microRNAs (miRNAs) or as long-intergenic noncoding RNAs (lincRNAs).

DNA methylation and hydroxymethylation

The predominant epigenetic tag in the DNA of differentiated mammalian cells is the covalent attachment of a methyl group to the C5 position of cytosine residues. This modification targets the dinucleotide sequence CpG [91;92]. DNA is methylated by DNA methyltransferases (DNMTs), resulting in the formation of 5-methylcytosine (5-mC). On the other hand, oxidation of 5-mC by the TET family of dio-oxygenase enzymes yields 5-hydroxymethylcytosine (5-hmC) [93;94]. Overall, increased abundance of 5-mc DNA is associated with transcriptional repression, whereas hypomethylation (i.e. less 5-mc and more 5-hmc) is linked with the activation of gene transcription [95;96]. The equilibrium of both tags at a defined genomic region will depend on DNMTs and TET enzymes activity. While DNMT1 is responsible of maintaining the basal levels of DNA methylation, DNMT3A and DNMT3B are in charge for *de novo* methylation of DNA that is either unmethylated or hemimethylated [91]. It's important to emphasize that both tags, 5-mc and 5-hmc, occur simultaneously throughout the genome. However, while 5-mc is more abundant in silenced genes and compacted chromosomal regions that display heterochromatin (i.e. closed-state or condensed chromatin), 5-hmc is associated with more accessible regions or euchromatin (i.e. open-state or exposed chromatin) at enriched promoter and/or enhancer regions of active genes [95].

Histone Post-Translational Modifications (PTMs)

Histones can be post-translationally modified to restructure the chromatin in multiple ways. PTMs occur on N-terminal tails of histones (H2A, H2B, H3, and H4) that make up the core of the nucleosome surrounded by two superhelix turns of DNA [97;98]. These tails are the most accessible region of the histone proteins and can be subjected to acetylation, methylation, phosphorylation, ubiquitination, and sumoylation among others [97]. PTMs can create new recruitment sites for specific factors, or alter the existing ones in order to eliminate previous interactions [99]. In addition, PTMs are capable to recruit enzymes that can 'write', 'erase' or 'read' modifications. There is a large repertoire of such histone modifiers (around 150 enzymes in humans) [100].

Acetylation and methylation of lysine at histone tails are the two most common PTMs, with distinct distributions along both heterochromatin and euchromatin [101]. Generally, acetylation is related to activation of gene transcription, and deacetylation with transcriptional repression [97]. Lysine acetylation is mediated by histone acetyltransferase enzymes (HATs) and deacetylation by histone deacetylases (HDACs) [97;102;103]. The positive charge of lysine residues binds strongly to the negatively charged DNA condensing nucleosomes and making chromatin inaccessible to transcription factors. Acetylation removes positive charges, reducing the affinity between DNA and histones, and exposing the compacted chromatin to give the transcriptional machinery an easier access to promoter/enhancer regions [102]. In contrast, histone methylation is related to either transcriptional activation or repression depending on where methylation takes place [101]. Different lysine residues can by modified by adding one, two or three methyl groups. The effect on the chromatin state and gene function will depend on the specific lysine modified and its degree of methylation. In general, methylation of lysine 9 and 27 of H3 (H3K9me and H3K27me)

is associated with transcriptional quiescence, whereas trimethylation of H3 at lysine 4 (H3K4me3) is mostly present at active promoter and enhancer regions [97;104].

Noncoding RNAs as epigenetic modulators

Contrary to former impressions, recent studies have showed that most of the human genome is transcribed into ncRNAs, instead of protein-encoding messenger (m)RNAs [105;106]. An astonishing variety of biological functions are accomplished by ncRNAs: they regulate gene expression at the levels of transcription, RNA processing and translation [107]. ncRNAs are classified in two main groups: small RNAs (sRNAs) and long noncoding RNAs (lncRNAs). sRNAs are 20–30 nucleotides long and lncRNAs are longer than 200 nucleotides [106;108]. Three types of sRNAs can be found: miRNAs [105;108], endo-small inhibitory RNAs (endo siRNAs) [109], and piwiRNAs (piRNAs) [110;111], all of them are implicated in epigenetic silencing [105]. The most well known in terms of epigenetic regulation are the miRNAs [112], since they target mRNAs encoding proteins required for both DNA methylation and histone PTMs [112]. Some of the miRNA targets include DNMTs, TETs, EZH2 (enzyme responsible for the H3K27me3 mark), and HDAC1, 4 and 6 [112].

The lncRNAs epigenetic contribution is reasonably more complex. They undergo polyadenylation despite they do not code for any protein [106], and most of them, named lincRNAs, are generated from long gene-free (i.e. intergenic) regions within the genome. These lincRNAs bind to chromatin-modifying complexes and guide them to genomic regions implicated in the control of gene expression [106;113].

Thousands of lincRNAs have been epigenetically identified in the genome because of two signature marks, H3K4me3 at regulatory regions of genes transcribed by RNApol II, together with the trimethylation of H3 at lysine 36 (H3K36me3) throughout the transcribed segment. Both marks result in a chromatin configuration known as bivalency [114].

Epigenetic mechanisms behind the hypothalamic control of reproductive development

Recent studies have highlighted the contribution of epigenetics at three levels of hypothalamic regulation; throughout the development of the GnRH neuronal network, within the AVPV kisspeptin neurons responsible of the preovulatory surge of gonadotropins and in ARC KNDy neurons responsible for the control of pulsatile GnRH secretion (**Summarized in** Table 1).

Sexual differentiation of the hypothalamus

Neuronal sex differences take place at many levels and are determined by steroid hormones. Testosterone from the gonads, converted into estradiol (E2) in the brain, is the main factor causing sexual differentiation, affecting adult reproductive physiology and behavior. The development of the preoptic area (POA), involved in sexual and maternal behavior, is strongly coordinated by E2. A number of epigenetic modifications within the POA, mostly DNA methylation and histone PTM patterns of the estrogen receptor alpha (ERα) gene have shown to influence the estrogen-dependent sexual differentiation of this region in rodents

[115]. While a segment of the ERα promoter displays a pattern of DNA methylation throughout development that does not correlate with ERα gene expression [116], another segment exhibits DNA methylation with a good correlation [117], suggesting that overall changes are subtle and confined, and only limited to some CpGs in specific portions of the 5′ flanking regulatory region of ERα. On the other hand, histone PTMs have a more crucial role than DNA methylation regarding the masculinization of the POA, since brain-targeted inhibition of HDAC activity in neonatal rats show a reduced male behavior when adults [118]. In addition, HDAC2 and HDAC4, apparently in association with ERα, are required for proper male behavior.

The expression of *Kiss1* in the AVPV area is sexually dimorphic [119]. Despite the fact that *Kiss1* expression is considerable higher in females than males [12], its promoter methylation levels are significantly larger in females [12]. This difference, although counterintuitive, may reflect the DNA methylation ability to block the recruitment of repressive transcriptional factors under certain conditions [12]

GnRH neuron maturation: DNA methylation and miRNA processing

Whole-Genome Bisulfite Sequencing (WGBS) studies together with RNA-sequencing (RNA-seq) performed on goat hypothalamus support the concept that gene expression patterns, influenced by changes in DNA methylation, regulate the onset of puberty [120]. Several genomic regions, including promoters, introns and untranslated regions (UTRs) have shown significant differences in expression of methylation profiles throughout development [120]. In addition, studies performed in non-human primate cultured GnRH neurons evidenced a decrease in methylation levels at multiple specific CpGs upstream the GNRH transcription starting site (TSS) throughout development, suggesting that demethylation at regulatory regions of the GNRH gene removes the repressive influence facilitating GNRH transcription [121]. In fact, GnRH mRNA levels increase significantly between 14 and 18 days in vitro, when the peptide release reaches a maximum, coinciding with decreasing DNA methylation.

In an elegant study, Messina and colleagues [122] determined the role of miRNAs as epigenetic regulators of the pubertal onset and reproductive maturation. By crossing Dicer-flox with GnRH-Cre animals, the authors generated a GnRH specific Dicer knockout mouse model, in order to determine the role of miRNAs in GnRH neuron maturation and reproductive competence. These mice showed impaired miRNA synthesis that lead to reduced plasma LH and FSH, hypogonadotropic hypogonadism and infertility due to a loss in GnRH expression. These animals displayed a reduction in transcriptional activation of the *Gnrh* gene and an increase in *Zeb-1* and *Cebpp*, two potent transcriptional repressors of *Gnrh*. Altogether, this study suggests that there is a multilayered miRNA-operated switch during the infantile-to-juvenile transition within GnRH neurons, where two key components, miR-200 and miR-155, regulate *Zeb1*, *Cebpb*, and *Gnrh* expression in order to modulate the balance between activating and repressive signals that ultimately regulate the neuroendocrine control of reproduction.

ARC Kiss1 and the onset of puberty

The presence of a transcriptional repressive mode of control regulating PA genes was first proposed by our studies of the TRG network whose central nodes were able to repress the transcriptional activity of *Kiss1* [73;77]. Subsequently, conclusive evidence demonstrated that the Polycomb Group (PcG) of transcriptional repressors prevents the premature initiation of puberty by inhibiting *Kiss1* transcription in KNDy neurons within the ARC [123]. Here, cDNA arrays and methylation array studies were performed on hypothalamic tissue during the prepubertal developmental phases in female rats. The three stages examined were: postnatal day (PND) 21 (early juvenile), PND 28 (late juvenile, the day when diurnal pulsatile LH is increased [124;125]) and PND 32–36 (late proestrus, the day of the first preovulatory surge of gonadotropins [126]). Using *Kiss1* as a prototype, it was shown that two central members of the PcG, *Eed* and *Cbx7,* are expressed within ARC kisspeptin neurons, and their protein products are associated to the *Kiss1* promoter during prepubertal development [123]. When the juvenile development period is approaching the end, the methylation of *Eed* and *Cbx7* promoter regions increase in the ARC at the same time the expression of both genes get significantly reduced. Interestingly, as *Eed* and *Cbx7* lower their expression, EED and CBX7 become evicted from the *Kiss1* promoter in an estrogen independent manner. Importantly, the loss of PcG components from the promoter is accompanied with a reorganization of the chromatin status, showing increased levels of the epigenetic marks H3K9ac, H3K14ac and H3K4me3, which are modifications associated with gene activation. Finally, and as predicted by these epigenetic changes, *Kiss1* mRNA expression is increased in the ARC.

The *Kiss1* promoter contains a bivalent region where both repressive and activating marks coexist. This allows the promoter to be poised for activation in response to diverse incoming inputs [127]. Despite the fact that the content of repressive H3K27me3 mark does not decrease at the same speed as the activating H3K4me3 mark increases, this repressive mark diminishes on the day of the pre-ovulatory surge of gonadotropins [128].

The fact that the *Kiss1* promoter is subjected to increasing epigenetic-activating marks like trimethylation and acetylation of H3 during pre-pubertal development denotes the activity of an activating transcriptional complex occurring simultaneously to the loss of PcG inhibition. The candidates for this activating role are members of the TrxG complex due to its well-known PcG-antagonizing activity [129;130] (Figure 1).

MLL1 and MLL3, two members of the TrxG complex, acting on promoter and enhancer regions of the *Kiss1* gene respectively, provide the trans-activational influence at the time when the inhibitory influence of the PcG complex is fading [128]. siRNA mediated ARC specific down regulation of Mll1 expression, inhibits *Kiss1* expression and delays puberty [128]. Furthermore, the use of a CRISPR-CAS9 epigenetic remodeling approach that inhibits the action of Mll3 on the *Kiss1* enhancer region, also blocks the peripubertal rise in Kiss1 expression and delays puberty [128]. In addition, the TrxG member UTX may assist the PcG eviction by demethylating the repressive histone H3K27me3 mark [131;132] allowing for an increase in H3K27ac, a hallmark of an active enhancer [128]. By implementing these essential key histone PTMs, the TrxG is capable of changing the epigenetic control of *Kiss1* from a repressive to an active state around the time of puberty

(Figure 1). A translational view for the role of TrxG in the control of puberty was evidenced by the fact that inactive mutations of CHD7, which in normal conditions antagonizes the PcG activity by binding to activating marks H3K4me2/me3 via its chromodomain, results in hypothalamic hypogonadism in humans [133].

Recently, our lab provided evidence that members of the zinc finger (ZNF) family of transcriptional repressors are also important components of the system that keeps the GnRH pulse generator in check during the pre-pubertal development, by preventing the premature re-awakening of GnRH secretion in non-human primates [134]. While *KISS1* and *TAC3* mRNA expression increases, *GATAD1* expression decreases in the MBH of both ovary-intact females and agonadal male rhesus monkeys during the juvenile-pubertal transition. In addition, when overexpressing *GATAD1* in the ARC of immature rats, the time of puberty becomes significantly delayed and the estrous cyclicity gets seriously compromised [134]. GATAD1, a well-known chromatin reader, recruits the histone eraser KDM1A, inducing the loss of activating H3K4me3/2 marks at gene regulatory regions [135;136]. Through *in vitro* studies, we showed that KDM1A recruitment increases when GATAD1 is overexpressed, and the association of H3K4me2 to both, *KISS1* and *TAC3* promoters gets significantly reduced. These results are consistent with the idea that GATAD1 attenuates *KISS1* and *TAC3* gene activity, in part by facilitating the loss of the H3K4me2 mark from PA gene promoters via gain of KDM1A (Figure 1). The decrease in GATAD1 and KDM1A association to the *KISS1* and *TAC3* promoters and the concomitant increase in H3K4me2 abundance observed in the monkey MBH at the time of puberty, strongly support the *in vitro* results and the notion that there is a mechanism of epigenetic repression that is lifted during the re-awakening of the GnRH pulsatility determined in the monkey infantile-juvenile transition.

Kiss1 activation and the preovulatory surge of gonadotrophins

It is known that both populations of kisspeptin neurons are differentially regulated by E2. While E2 inhibits *Kiss1* expression in KNDy neurons of the ARC, it enhances its expression in kisspeptin neurons of the AVPV. The mechanisms involved in this differential effect are not yet understood. Recent evidence has showed that E2 has an epigenetic role in the control of kisspeptin neurons within the AVPV [137]. Here, E2 induces acetylation of H3 at the promoter region of *Kiss1,* increasing its expression. In contrast, the acetylation of H3 is significantly reduced in ARC *Kiss1* regulatory region and therefore *Kiss1* expression is reduced [137]. In addition, E2 induces ERα binding to the *Kiss1* promoter exclusively in the AVPV area. Because no changes were observed in the DNA methylation pattern of *Kiss1* promoter either in the AVPV or ARC, it is suggested that DNA methylation does not contribute as an epigenetic cue to the modulation of *Kiss1* expression at the time when the preovulatory surge of gonadotrophins takes place. Surprisingly, an estrogen-responsive enhancer region located in the intergenic 3′ region of *Kiss1* gene was discovered in kisspeptin neurons from the AVPV but not from the ARC [137]. Despite these results, unveiling an epigenetic contribution for E2 positive feedback in the AVPV, it is not clear whether there is a similar epigenetic mechanism for estrogen inhibitory action on ARC *Kiss1* expression (Figure 2).

Genomic imprinting and mutations at the Makorin 3 (MKRN3) gene

Recent studies [138–148], have found heterozygous loss of function mutations of MKRN3 in patients with central precocious puberty. Moreover, GWAS found that a specific SNP near the paternal allele of the *MKRN3* gene is associated with earlier age at menarche [149;150]. *MKRN3* is an intronless gene located in the Prader-Willy syndrome region of chromosome 15q11.2. and is maternally imprinted, meaning that patients inherit the mutation from their paternal allele [138]. A gender dimorphism appears evident since MKRN3 mutations seem to affect females more severely than males [138;139]. *Mkrn3*, which has only been identified in therian mammals, belongs to a highly conserved family of makorin genes [151]. Makorins are zinc finger proteins with C3HC4 motifs called RING domains associated with E3 ubiquitin ligase activity [151]. These enzymes are involved in transferring ubiquiting from an E2-ubiquiting-conjugating enzyme to target proteins. Makorins are also implicated in the regulation of RNA polymerase II activity, suggesting they could be involved in transcriptional and/or epigenetic modes of regulation [152]. The exact mechanism by which MKRN3 mutations affect the timing of puberty is currently unknown, but *Mkrn3* is highly expressed in the ARC nucleus of juvenile mice, and its expression decreases as puberty progresses, suggesting that it functions as a strong repressor of the GnRH network [138]. More research is needed to verify the role of this gene or the involvement of genomic imprinting in the pathogenesis of central precocious puberty.

Conclusions

Nowadays it is well established that epigenetics plays a major role in the regulation of reproductive development and the initiation puberty. The interaction between epigenetic activation and repression of gene transcription seems to be the core of a process by which epigenetic mechanisms are able to modulate the development of the pubertal process. Much more remains to be unveiled in order to characterize components and comprehend signal pathways triggered by epigenetic mechanisms that regulate gene expression in a coordinated fashion throughout prepubertal neuroendocrine development. Moreover, studies that identify how epigenetic pathways convey information from a wide range of stimuli (e.g. nutrition, environment, circadian activity) to hypothalamic neurons regulating the onset of puberty are still in need. For that, it is important to emphasize that the epigenetic code, linking DNA methylation, histone PTMs and ncRNAs is extremely dynamic and likely to diverge from different cell populations and at different developmental times. New technologies and methods available, like the possibility of isolating specific cellular types from the neuroendocrine hypothalamus together with micro methods for genome-wide characterization of histone landscapes and/or DNA methylation profiles, will help determine the epigenetic status of defined cell populations at specific times of development, or after the exposure to stimuli.

While this research is still in its infancy, we have made great advances to the field by opening new avenues in our understanding of this complex and multilayered developmental process. We envision the future use of epigenetic biomarkers as a readout of normal developmental patterns that could also serve as early detectors of deranged pubertal development.

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Acronyms used

5-mC	5-Methylcytosine
5-hmC	5-Hydroxymethylcytosine
ARC	Arcuate Nucleus
ATP	Adenosine Triphosphate
AVPV	Anteroventral Periventricular Nucleus
CBX7	Chromobox Homolog 7
Cebpb	CCAAT/Enhancer-Binding Protein Beta
CHD7	Chromodomain Helicase DNA Binding Protein 7
DNA	Deoxyribonucleic Acid
DNMT1	DNA Methyltransferase 1
DNMT3A	DNA Methyltransferase 3A
DNMT3B	DNA Methyltransferase 3B
E2	Estradiol
EAP1	Enhanced at Puberty 1
EED	Embryonic Ectoderm Development
ERα	Estrogen Receptor Alpha
erbB4	Erb-B2 Receptor Tyrosine Kinase 4
EZH2	Enhancer of Zeste 2
FSH	Follicle-Stimulating Hormone
GABA	Gamma-Aminobutyric Acid
GAB2	Growth Factor Receptor Bound Protein 2-Associated Protein 2
GAD67	Glutamate Decarboxylase 67
GATAD1	GATA Zinc Finger Domain Containing 1
GLS	Glutaminase
GnIH	Gonadotropin-Inhibitory Hormone

GnRH	Gonadotropin-Releasing Hormone
GWAS	Genome Wide Association Study
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
H3K4me3	Histone 3 Trimethylated at Lysine 4
H3K9ac	Histone 3 Acetylated at Lysine 9
H3K14ac	Histone 3 Acetylated at Lysine 14
H3K9me1	Histone 3 Monomethylated at Lysine 9
H3K9me2	Histone 3 Dimethylated at Lysine 9
H3K27ac	Histone 3 Acetylated at Lysine 27
H3K27me	Histone 3 Monomethylated at Lysine 27
H3K36me3	Histone 3 Trimethylated at Lysine 36
HATs	Histone Acetyltransferase Enzymes
HDAC1	Histone Deacetylase 1
HDAC2	Histone Deacetylase 2
HDAC4	Histone Deacetylase 4
HDAC6	Histone Deacetylase 6
KDM1A	Lysine Demethylase 1A
KDM3B	Lysine Demethylase 3B
KISS1	KiSS-1 Metastasis-Suppressor
KISS1R	KISS1 Receptor
KNDy	Kisspeptin/Neuokinin B/Dynorphin
LH	Luteinizing Hormone
lncRNA	Long-Noncoding RNA
lincRNAs	Long-Intergenic Noncoding RNA
MKRN3	Makorin Ring Finger Protein 3
mRNA	Messenger RNA

miRNA	MicroRNA
Mll1	Mixed-Lineage Leukemia 1
Mll3	Mixed-Lineage Leukemia 3
NELL2	Neural Epidermal Growth Factor-Like Like 2
ncRNA	Non-Coding RNA
NKB	Neurokinin B
NR4A2	Nuclear Receptor Subfamily 4 Group A Member 2
NRG1	Neuregulin-1
OCT2	Organic Cation Transporter-2
PA	Puberty Activating
PAX8	Paired Box 8
PDYN	Prodynorphin
PGE2	Prostaglandin E2
PI	Puberty Inhibitory
piRNA	PiwiRNA
PcG	Polycomb Group
PND	Postnatal Day
POA	Pre-Optic Area
PTM	Posttranslational Modifications
RFamide	Arginine and Amidated Phenylalanine
RING	Really Interesting New Gene
RNA	Ribonucleic Acid
RNA-seq	RNA-sequencing
RNApol II	RNA polymerase II
SNP	Single-Nucleotide Polymorphism
sRNA	Small RNA
SynCAM	Synaptic Cell Adhesion Molecule
TAC3	Tachykinin 3
TET	Ten-Eleven Translocation Methylcytosine Dioxygenase

TMEM18	Transmembrane Protein 18
TRG	Tumor Related Genes
TrxG	Trithorax Group
TSLC1	Tumor-Suppressor Gene in Human Non-Small-Cell Lung Cancer
TTF1	Transcription Termination Factor 1
UTR	Untranslated Regions
UTX	Ubiquitously Transcribed Tetratricopeptide Repeat-X
WGBS	Whole-Genome Bisulfite Sequencing
Zeb1	Zinc Finger E-Box Binding Homeobox 1
ZNF462	Zinc Finger Protein 462
ZNF483	Zinc Finger Protein 483

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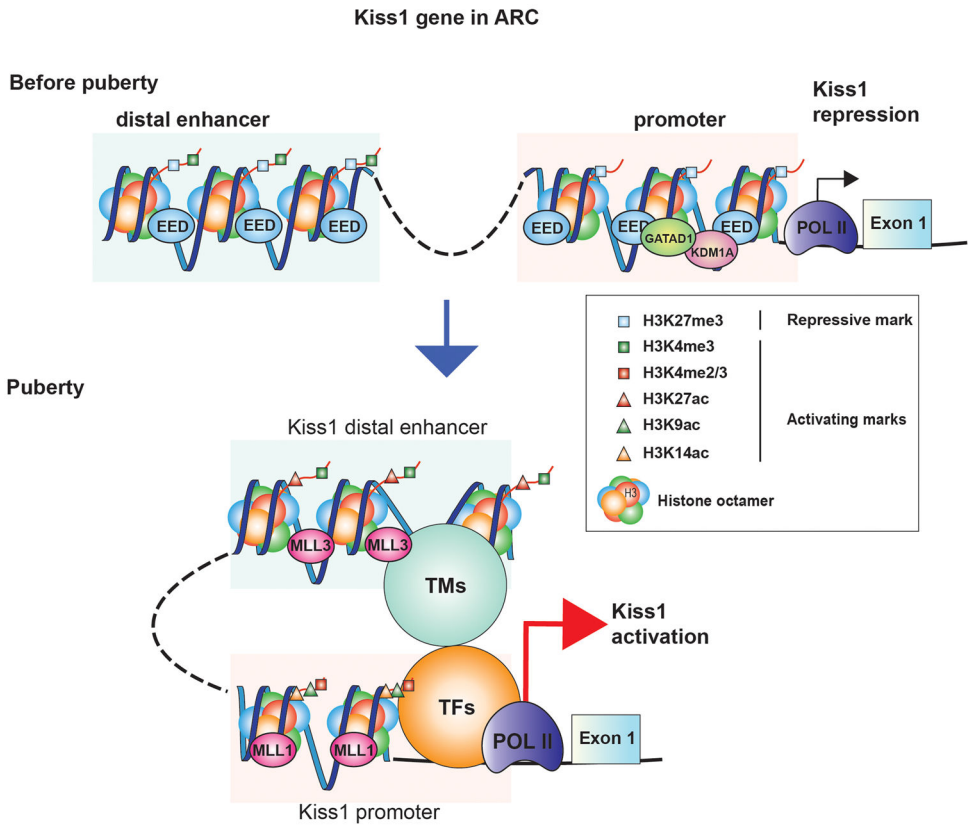

Figure 1. The epigenetic control of *Kiss1* expression in the ARC

This model postulates that during prepubertal development the transcriptional activity of the *Kiss1* gene is repressed by a series of repressors, exemplified by the PcG as well as the GATAD1/KDM1A complex. By depositing histone PTMs associated with gene silencing (e.g. H3K27me3, blue squares) at *Kiss1* promoter and enhancer sites, PcG proteins (epitomized by EED) act as 'writers' of a repressive chromatin configuration. Furthermore, the GATAD1 'reader' protein recruits KDM1A, the 'eraser' of H3K4me2 further repressing chromatin accessibility. As puberty advances, these 'writers' and 'readers' are removed from these regulatory regions, and as a consequence of this loss, the amount of H3K27me3 is reduced, while H3K4me2/3 increases. As this transformation takes place, another set of writers, typified by the TrxG-activating multiprotein complex is engaged causing the deposition of histone PTMs linked to transcriptional activation at two sites: a) H3K4me2/3 (red squares) and H3K9,14ac (green and orange triangles) are added at promoter regions and, b) H3K4me3 and H3K27ac (red triangles) at enhancer sites characterized by their high and sustained content in H3K4me1 (green squares). This chromatin looping also brings a series of unknown transcription factors (TFs) like pioneer, determining and collaborative TFs, as well as transcriptional mediators (TMs) like Histone Acetyltransferases, DNA demethylation enzymes, Chromosomal looping factors, RNA processing factors, non-coding RNAs, etc [153]. An outcome of these changes is that the activated enhancer is attracted to the promoter region enhancing polymerase II (POLII) transcriptional activity, thus increasing *Kiss1* mRNA transcription.

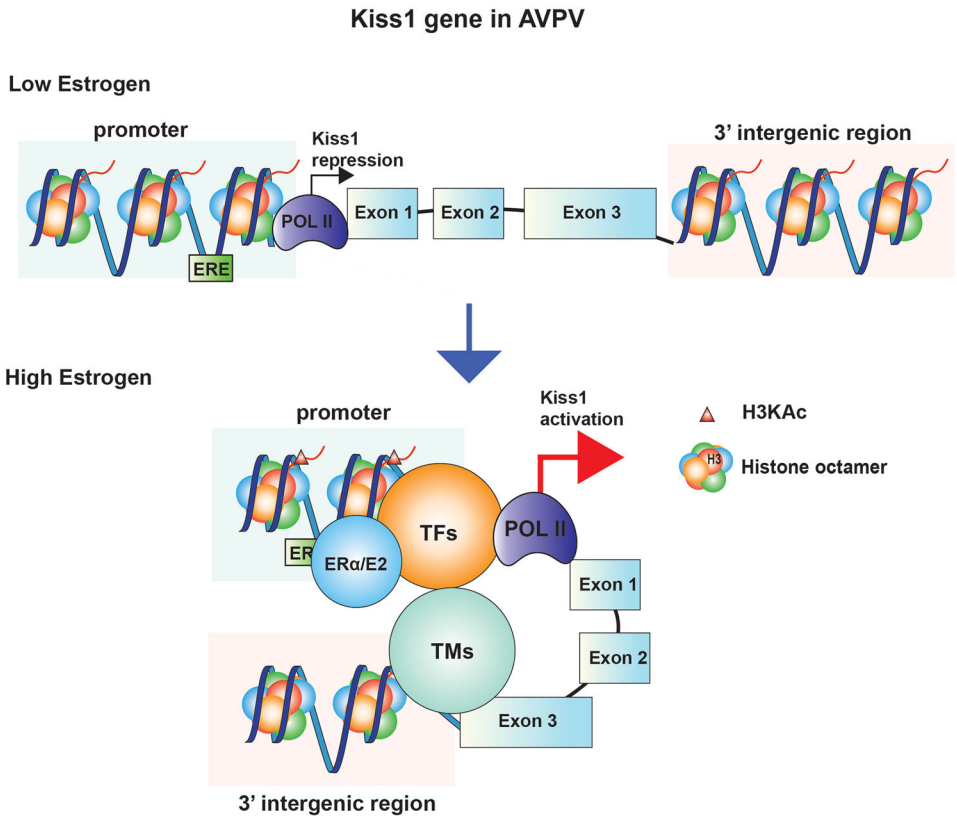

Figure 2. The epigenetic control of *Kiss1* expression in the AVPV

During prepubertal development the transcriptional activity of the *Kiss1* gene in the AVPV is repressed. After ARC kisspeptin induced GnRH pulsatilitiy and thus LH release is increased, ovarian maturation takes place increasing estrogen production. When serum estrogen reaches pre-ovulatory levels, AVPV kisspeptin expression is induced trough ERα binding to ERE sites (green rectangles), enhancing Histone 3 acetylation (red triangles), as well as formation of 3D chromatin rearrangements bringing transcription factors (TFs) and transcriptional mediators (TMs) like: Histone Acetyltransferases, DNA demethylation enzymes, Chromosomal looping factors, RNA processing factors, non-coding RNAs, etc [153] in close proximity to the RNA polymerase 2 (Pol II).

Table 1

Epigenetic enzymes characterized in the hypothalamic control of pubertal development.

Enzyme	Function	Epigenetic mark	Target	Effect on normal puberty	Reference
DNMTs	Writer	CpG methylation	GnRH	Repression	[117]
DNMTs	Writer	CpG methylation	Cbx7 and Eed promoters	Activation	[123]
DICER	miRNA's processing	miR-200 miR-155	Zeb1/GnRH Cebpb	Activation	[122]
Polycomb (EED and CBX7)	Writer	H3K27me3	Kiss1 promoter	Repression	[123]
GATAD1	Reader	H3K4me2	Kiss1 promoter	Repression	[134]
KDM1	Eraser	H3K4me2	Kiss1 promoter	Repression	[134]
MLL1	Writer	H3K4me3	Kiss1 promoter	Activation	[128]
HATs	Writer	H3K9ac H3K14ac	Kiss1 promoter	Activation	[123]
MLL3	Writer	H3K4me3	Kiss1 enhancer	Activation	[128]
P300/CBP	Writer	H3K27ac	Kiss1 enhancer	Activation	[128]