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Mini-Review

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Emerging Roles of Epigenetics in the Control of Reproductive Function: Focus on Central Neuroendocrine Mechanisms

Maria Jesus Vazquez,[1,2,3] Silvia Daza-Dueñas,[1,2] and Manuel Tena-Sempere[1,2,3,4,5]

[1]Instituto Maimónides de Investigación Biomédica de Cordoba (IMIBIC), 14004 Cordoba, Spain; [2]Department of Cell Biology, Physiology and Immunology, University of Cordoba, 14004 Cordoba, Spain; [3]Hospital Universitario Reina Sofia, 14004 Cordoba, Spain; [4]CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, 14004 Cordoba, Spain; and [5]Institute of Biomedicine, University of Turku, FIN-20520 Turku, Finland

ORCiD numbers: 0000-0002-5350-4830 (S. Daza-Dueñas); 0000-0002-4741-5567 (M. Tena-Sempere); 0000-0001-9642-4024 (M. J. Vazquez).

Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mc, 5-methylcytosine; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BPA, bisphenol A; DNMT, DNA methyltransferase; EDC, endocrine disruptor; ER, estrogen receptor; H, histone; HDAC, histone deacetylase; HPG, hypothalamic-pituitary-gonadal; MBH, mediobasal hypothalamus; MLL, mixed-lineage leukemia; NKB, Neurokinin B; PcG, Poly-comb group; PCOS, polycystic ovary syndrome; TET, ten-eleven translocation; TrxG, Trithorax group; UTR, untranslated region; ZNF, zinc finger motif

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Abstract

Reproduction is an essential function for perpetuation of the species. As such, it is controlled by sophisticated regulatory mechanisms that allow a perfect match between environmental conditions and internal cues to ensure adequate pubertal maturation and achievement of reproductive capacity. Besides *classical* genetic regulatory events, mounting evidence has documented that different epigenetic mechanisms operate at different levels of the reproductive axis to finely tune the development and function of this complex neuroendocrine system along the lifespan. In this mini-review, we summarize recent evidence on the role of epigenetics in the control of reproduction, with special focus on the modulation of the central components of this axis. Particular attention will be paid to the epigenetic control of puberty and Kiss1 neurons because major developments have taken place in this domain recently. In addition, the putative role of central epigenetic mechanisms in mediating the influence of nutritional and environmental cues on reproductive function will be discussed.

Key Words: epigenetics, puberty, reproduction, Kiss1, kisspeptins, GnRH, nutrition, environmental cues

As is the case for other essential bodily functions, reproduction is governed by a highly hierarchical neuroendocrine axis, in which its major hormonal signals are connected via feed-forward and feed-back regulatory loops. This is driven by the hypothalamic-pituitary-gonadal (HPG) axis, whose upper element is the decapeptide, GnRH, secreted

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in a pulsatile manner by a scarce population of GnRH neurons located in the basal forebrain. GnRH reaches the pituitary via the hypothalamic-pituitary portal vasculature to stimulate the secretion of both gonadotropins, LH and FSH. In turn, gonadotropins operate on the gonads to induce potent trophic effects, generate gametes, and promote the release of sex steroids and peptides [1, 2]. Although this axis undergoes dramatic changes during the lifespan, a major maturational period is puberty, when the secretory activity of the elements of the HPG axis becomes fully activated. Although some aspects of the HPG axis are conserved between sexes (eg, the negative feedback effects of gonadal steroids and the tonic mode of pulsatile secretion of gonadotropins), functioning of the axis displays clear sex differences, with positive feedback actions of ovarian steroids and a surge-mode secretion of gonadotropins as the driver of ovulation, occurring only in females [3].

As a reflection of its highly hierarchical role, GnRH neurons, located in the rostral hypothalamic area in mammals, are responsible for the dynamic integration of a wide array of central and peripheral signals, including circulating hormones, neuropeptides, and neurotransmitters, which permit the precise regulation of the HPG axis at different developmental stages and therefore play a fundamental role in the control of puberty onset and reproductive function later in life [4]. The sophisticated nature of the mechanisms controlling GnRH neurosecretion is well illustrated by the pubertal transition, when a change in the balance between stimulatory and inhibitory afferents results in a switch in the neurosecretory activity of GnRH neurons, leading to a predominant activatory program that leads to complete awakening of the HPG axis and acquisition of reproductive capacity [2]. Moreover, at early developmental (gestational) stages, GnRH neurons undergo a complex maturational program, involving the migration of GnRH neurons from the olfactory placode to the hypothalamus, which is an absolute prerequisite for proper organization of the HPG axis, its activation at puberty, and later function in adulthood [5].

Evidence from clinical and experimental studies have solidly documented the importance of genetic determinants of reproduction, as illustrated by an ever-growing number of gene mutations known to have a deleterious effect in either developmental or regulatory aspects of the HPG axis [6]. This epitomized by the impact of known mutations affecting either the migration or the function of GnRH neurons. In fact, the advent of next-generation sequencing has allowed further expansion of our understanding of the genetic basis of reproductive control because of the identification of major controllers of puberty and fertility. Likewise, very solid evidence has demonstrated the crucial role of transcriptional regulatory events of major modulators of the HPG axis, such as those conducted by sex steroids, whose fundamental role in reproductive regulation has been well defined [7]. However, compelling evidence gathered mainly in the past decade has documented that epigenetic regulatory mechanisms, acting at different levels of the HPG axis, are also involved in the control of key aspects of reproductive maturation and function [8, 9]. In this review, we summarize major developments in this domain, with a particular focus on central regulatory events and the modulation of puberty onset and Kiss1 neurons.

Key Players in Neuroendocrine Control of Reproduction: Fundamental Roles of Kisspeptins

The central regulatory mechanisms governing reproductive function, and particularly GnRH neurosecretory activity, are intricate and involve multiple regulatory signals. Among those, kisspeptins have emerged in the past 2 decades as very potent elicitors and master regulators of GnRH neurons [10]. Kisspeptins are encoded by the *Kiss1* gene and operate via the G-protein coupled receptor, Gpr54 (aka Kiss1R). Although exhaustive recapitulation of the physiological roles of the so-called Kiss1 system is beyond the scope of this mini-review, and has been addressed elsewhere [10, 11], a brief summary of its major features is warranted, not only because of its paramount importance in the central control of puberty and fertility, but also because compelling evidence accumulated in recent years has pointed out that epigenetic mechanisms operating in Kiss1 neurons and affecting Kiss1 expression are likely to play a major role in reproductive control, especially as it pertains to pubertal control [12, 13].

In mammals, 2 main populations of Kiss1 neurons have been described in the hypothalamus: one located in the arcuate nucleus (ARC), or its equivalent infundibular region in humans, and the other placed in the rostral hypothalamic area, mainly at the anteroventral periventricular nucleus (AVPV), as has been well characterized in rodents [10]. Both populations, named hereafter as ARC and AVPV Kiss1 neurons, produce kisspeptin and are capable of activating GnRH secretion. Yet, they display remarkable sex and functional differences, are divergently modulated by key regulators (eg, sex steroids), and control different aspects of reproductive function [10].

The differential regulation of Kiss1 neuronal populations by sex steroids is a determinant for major functional features of the HPG axis. In rodents, estrogens upregulate Kiss1 expression in the AVPV population, which is far more prominent in females [14]. This is thought to be the basis for positive feedback effects of estradiol, which is mandatory for the preovulatory surge of gonadotropins as

major hormonal drivers of ovulation. Contrary, ARC Kiss1 neurons mediate the negative feedback of sex steroids by virtue of their capacity to suppress Kiss1 expression at this site, thereby contributing to shape the pulsatile secretion of gonadotropins in both females and males. In fact, recent evidence documents that ARC Kiss1 neurons are a major component of the so-called GnRH pulse generator [15]. Of note, a set of Kiss1 neurons in the ARC, named KNDy neurons [16], coexpress Neurokinin B (NKB) and Dynorphin. Studies conducted in rodents and other mammalian species have pointed out that both cotransmitters modulate kisspeptin output by ARC Kiss1 neurons in a reciprocal manner: while NKB stimulates kisspeptin secretion, Dynorphin inhibits it [17]. This oscillatory circuit seems crucial for the proper shaping of kisspeptin output to GnRH nerve terminals and hence for GnRH/gonadotropin pulsatility. Accordingly, KNDy neurons have been recognized as key elements in the central control of the HPG axis, capable of integrating metabolic and environmental signals to modulate GnRH activity and, thereby, timing of puberty and fertility. Interestingly, however, not all ARC Kiss1 neurons seem to be KNDy, and it has been suggested that a variable percentage of Kiss1-only and KNDY neurons may exist depending on the sex and species [18]. All in all, these features illustrate the complexity of the regulatory mechanisms controlling the development and function of these key Kiss1 neuronal populations, at different hypothalamic sites, that are possibly multifaceted and need to be further exposed.

Beyond Gene Sequences: Basic Epigenetic Regulatory Mechanisms

In addition to the information encoded by the mere nucleotide sequence of genes, it has become clear that important changes in gene expression, either transient or transmissible, are dictated by regulatory events that operate "upon" the genes, therefore defining a novel layer of modulation, defined as epigenetics [19]. This term refers to the whole set of heritable or reversible changes in gene expression/activity that do not involve changes in the primary nucleotide sequence and imply phenotype alterations without genotype modifications. These epigenetic variations can either be spontaneous or induced by internal or external cues and are fundamental for establishing cell diversity. As such, deregulation of epigenetic phenomena can result in pathological conditions [20, 21]. Notably, over the past decade, a growing number of studies has revealed the role of epigenetic regulation in mammals during fundamental processes throughout the lifespan, including reproductive development and the timing of puberty [22, 23]. By way of introduction for later sections, we briefly summarize the major epigenetic regulatory mechanisms operating in mammals.

The epigenetic regulation of gene expression can occur at 3 main levels: genomic DNA, chromatin structure, and ncRNAs (Fig. 1). Accordingly, 3 major forms of epigenetic control have been identified, as defined by chemical alterations of DNA, posttranslational modifications of histones, and regulation by ncRNA, of which small ncRNAs, known as miRNAs, are the best studied. Notably, these epigenetic mechanisms are often not disconnected events and can functionally interact to regulate transcriptional activity and protein translation in a coordinated manner.

DNA Modifications

The best-described chemical alteration of DNA in mammalian cells is methylation, which involves a covalent addition of a methyl group (CH_3) to position 5′ of a cytosine residue. This attachment is especially abundant in the dinucleotide sequence CpG (cytosine linked to guanine by a phosphate), although, non-CpG methylation has also been described in mammalian nervous system and embryonic stem cells. CpG sequences are broadly found in gene promoters, and their modifications are directly related to the regulation of gene expression. Thus, an increase of CpG methylation in gene promoters usually denotes inactivation, with chromatin in a closed state (heterochromatin) blocking gene expression.

DNA is methylated by DNA methyltransferases (DNMT), resulting in formation of 5-methylcytosine (5mC). There are different types of DNMTs: DNMT1, which maintains normal levels of methylation; DNMT2, associated with RNA methylation; and DNMT3a and DNMT3b, which are involved in de novo DNA methylation at CpG sites. On the other hand, the ten-eleven translocation (TET) family of enzymes, with dioxygenase activity, are responsible for oxidizing 5mC to yield 5-hydroxymethylcytosine (5hmC). The balance between both tags (5mC/5hmC) in a specific region is kept by DNMT and TET activity, indicative of transcriptional activity. Thus, an increase of 5mC DNA is associated with transcriptional gene repression (silenced genes), whereas predominance of 5hmC is an epigenetic modification related to transcriptional gene induction (active genes) [24]. Additionally, DNA modifications may be induced by transcriptional repressors containing zinc finger motifs (ZNFs). These ZNF proteins interact with DNA at specific regulatory sequences to repress gene expression [25].

Histone Posttranslational Modifications

The term chromatin refers to the association of DNA and sets of proteins, termed histones (H), that form functional complexes. The fundamental repeating unit of chromatin structure, the nucleosome, consists of DNA wrapped around a histone core, composed by H2A, H2B, H3, and

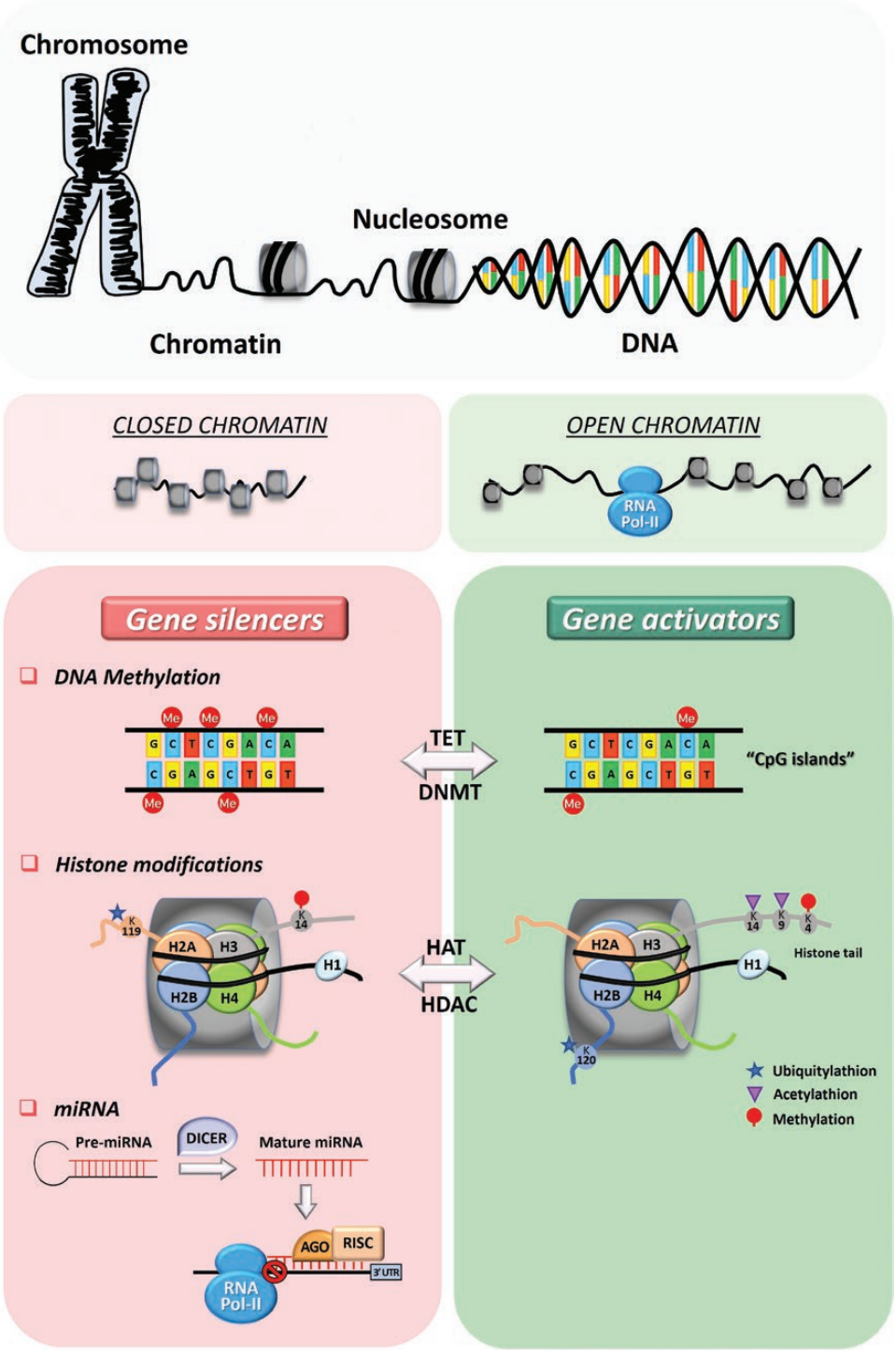

Figure 1. Basic epigenetic mechanisms for the control of gene expression. A schematic of the major epigenetic phenomena affecting gene expression is presented. Epigenetic mechanisms operate "upon" the DNA sequence to modulate gene expression, either by methylation of CpG islands or by histone posttranslational modifications. Repressive mechanisms are indicated on the left, whereas activator events are depicted on the right. Suppressive mechanisms involve also the action of miRNAs, which can promote mRNA degradation or block translation. For further details, see the text.

H4 [26]. Contiguous nucleosomes are linked by histones (H1) and other nonhistone proteins. Histones are key elements to maintain the structure and packaging of whole chromatin, thereby influencing gene expression.

Posttranslational modifications of histones are alterations of different residues, mainly occurring at their N-terminus tails. Potential modifications include acetylation, methylation, phosphorylation, ubiquitination, and

SUMOylation [27]. Although all these variations may influence DNA transcription, the addition/removal of methyl and acetyl groups on histones is, together with DNA methylation, the main mechanism of changing the epigenetic landscape [28]. The acetylation of histones consists of the addition of an acetyl group ($COCH_3$) to lysine residues on histone tails, and is carried out by histone acetyltransferases, whereas histone deacetylases (HDACs) are those responsible for removal of acetyl groups. The relative abundance of acetylated/deacetylated histones induces structural chromatin changes that alter the access of the transcriptional machinery to DNA. Thus, histone acetylation usually induces an open-state chromatin (euchromatin) that facilitates gene expression, whereas histone deacetylation is associated with a close-state chromatin (heterochromatin) and gene silencing [27, 29].

In turn, histone methylation is mediated by histone methyltransferases, and leads to the addition of a methyl group at lysine or arginine residues. However, its impact on gene expression is dual; thus, although H3 methylation at Lys 9 and 27 (H3K9me and H3K27me) is usually found in silenced genes, thererfore representing a repressive mark, trimethylation of H3 at Lys 4 (H3K4me3) is a signal of open chromatin and, hence, of gene transcription [27]. In this context, emerging evidence indicates the relevant role of 2 different histone methylating complexes, composed of chromatin proteins, with mutually antagonistic activity; the Poly-comb group (PcG) and the Trithorax group (TrxG). These drive transcriptional repression or activation primarily via their histone methyltransferase activity: PcG operates as repressor of gene transcription, whereas TrxG is considered to function as gene expression activator, antagonizing PcG-mediated silencing [30, 31].

Other forms of histone posttranslational modifications include ubiquitination of lysine residues in histone tails, which may result in either an open or close chromatin status. In fact, ubiquitination of histone 2A and 2B has been shown to participate in the control of the activity of PcG and TrxG groups, respectively [27]. The relevance of other histone modifications is not completely understood. In any case, histone phosphorylation at lysine and threonine residues has been related to gene activation, whereas histone SUMOylation has been shown to play an important action controlling steroid receptor activity, which is especially relevant in cancer.

nc RNA: Prominent Roles of miRNAs

It is well established that the genome, including that of humans, is transcribed into mRNAs, coding for proteins, and a wide array of ncRNAs. There are 2 main groups of ncRNA based on their length: long ncRNA (> 200 nucleotides) and small ncRNA (~20 to 30 nt) [32]. Among the different classes of small ncRNA, miRNAs are the most studied and the best known to participate in the regulation of the expression/translation of a broad number of genes [33]. During their biogenesis, the precursor miRNAs (pre-miRNAs) are exported to the cytoplasm, where the endonuclease, DICER, splits the hairpin pre-miRNA to form double-stranded miRNA. Final miRNA maturation implies the dissociation and degradation of one strand, whereas the other (miRNA guide) is incorporated into Argonaute proteins and RNA-induced silencing complex. By complete or partial complementarity, the subsequent complex recognizes specific seed regions, usually located at 3′-untranslated region (UTR), in target mRNAs and, in most cases, induces gene silencing, either by preventing protein translation or promoting mRNA degradation [34]. However, the mechanisms underlying miRNA activity are more complex; thus, a single miRNA can target different genes simultaneously and may be regulated in a tissue-specific manner. In addition, some miRNAs may recognize sequences located in positions other than the 3′-UTR, such as the coding or the 5′-UTR region of target genes [34]. Rather unusually, some miRNAs can promote gene expression, rather than silencing.

Other ncRNA may participate also in epigenetic regulation, albeit their function is not fully understood. This is the case of long ncRNA, which can interact directly with chromatin-modifying complexes, act as guides to recruit them to specific DNA regulatory sites, or even interact with other ncRNAs, as is the case of miRNAs. It has been reported that long ncRNA can bind/sequester miRNAs, avoiding their interaction with the corresponding targets [35].

Major Mechanisms of Epigenetic Control of Reproduction: Focus on Central Aspects

Compelling evidence, accumulated mostly over the past 2 decades, has documented a prominent role of epigenetics in the fine control of development and function of the reproductive system. Epigenetically controlled events range from sexual determination and gonadogenesis to brain sexual differentiation and the dynamic regulation of key reproductive events, including pubertal maturation and GnRH neurosecretion [30, 36]. Importantly, epigenetic regulatory mechanisms are at the core of reproductive adaptations to different environmental and developmental conditions [9] and thus can be the basis of some alterations of sexual development and reproductive function [36]. Epigenetic mechanisms are known to operate at all levels of the HPG axis, at different developmental windows, as illustrated by the importance of chromatin changes in early programing

events in germ cells [37], or the key role of an epigenetic switch leading to repression of TET1 in gonadotropes for activation of LH-beta expression and acquisition of reproductive competence [38]. Notwithstanding, in this review, we focus on brain epigenetic regulatory pathways, with a particular emphasis on pubertal maturation because this key maturational period has been shown to be tightly controlled by different epigenetic mechanism. In addition, the role of central epigenetic modifications in the early maturation (eg, sexual differentiation of the brain) and adult function of the reproductive axis will be also discussed here.

Epigenetic Regulation of Early Maturational Events

Epigenetic regulatory mechanisms are seemingly involved in key early maturational phenomena affecting brain sex differentiation and development of the reproductive axis [13]. Thus, rodent studies have demonstrated that changes in DNA methylation and histone modifications during the fetal and perinatal critical periods strongly influence estrogen-dependent sexual differentiation of the brain [13, 39]. In particular, pharmacological perturbation of the normal dynamics of histone acetylation/deacetylation during the critical postnatal period, by treatment with an inhibitor of HDAC that maintains elevated levels of histone acetylation, results in perturbed sex differentiation of specific brain areas, such as the bed nucleus of stria terminalis, and the volume of vasopressin fibers [13, 39, 40]. Likewise, pharmacological inhibition of HDAC activity specifically in the preoptic area of neonatal rats was able to disrupt adult male behavior [41]. On the other hand, changes in DNA methylation in early developmental periods are likely involved also in brain sex differentiation phenomena, as reflected by the fact that the level of expression of sexually differentiated genes, such as those encoding sex steroid receptors, in the hypothalamus correlate with the DNA methylation status in this brain region [42].

In addition, a series of studies by the Kauffman group addressed some years ago the potential of epigenetic regulatory mechanisms in the control of key aspects of Kiss1 neuronal maturation, and particularly the generation of sex differences in the AVPV population, which is larger in females [14]. Those studies documented that neonatal inhibition of HDAC resulted in an elevation of the number of Kiss1 neurons in the AVPV in both sexes, but did not obliterate sex differences, suggesting that histone deacetylation might participate in the development of the rostral population of Kiss1 neurons but is not mandatory for its sex differences (ie, females >> males) [22]. On the other hand, by exploring the methylation status of the Kiss1 promoter in the AVPV of male and female mice, the same authors were able to identify a number of CpG islands with differential methylation between males and females, with an overall trend for higher methylation in females [22]. Although this phenomenon might be suggestive of a role of changes in DNA methylation in the emergence of sex differences in Kiss1 neurons in the AVPV, the actual physiological relevance and mechanism underlying such phenomenon remains unknown.

In the same vein, changes in the methylation status of various 5′ CpG islands of the GnRH gene have been reported in cultures of immature GnRH neurons from the nasal placode of rhesus monkeys [43]. In detail, during early maturation in vitro, there was an increase of GnRH mRNA expression that was paralleled by a decrease in the methylation of 8 CpG sites of its promoter, therefore suggesting a potential role of DNA de-methylation in the enhancement of GnRH expression at early maturational stages of GnRH neurons. In line with these findings, studies in mice suggested that gene expression of the member of TET enzyme family, controlling DNA methylation, Tet2, is enhanced in the preoptic area during postnatal maturation. In addition, elevated Tet2 levels were found to increase of GnRH secretory activity in the immortalized mature mouse hypothalamic GnRH neuronal cell line, GT1-7, and seem to contribute to the maintenance of GnRH neurons, especially in the male [44].

Epigenetic Regulation of Puberty Onset

Substantial advancements have taken place during the past decade regarding our knowledge of the epigenetic mechanisms that control puberty onset. These include changes in DNA methylation of the promoters of key pubertal repressors, histone modifications, and changes in the chromatin landscape, as well as regulatory actions of miRNAs, which have been shown to target key neuronal populations of the reproductive brain, such as Kiss1 and GnRH neurons.

Roles of chromatin modifications and DNA methylation in the central control of puberty

Puberty onset is the result of a delicate balance of regulatory inputs, influenced by earlier maturational events and subjected to the control of multiple endogenous and environmental cues. Compelling evidence has documented that epigenetics provides an additional layer of regulation to the mechanisms governing pubertal maturation. In this context, the pioneering work of Ojeda's group has helped elucidate the molecular basis for the epigenetic control of puberty, defined by the reciprocal activity of 2 major families of transcriptional regulators, with either repressive or activating function, that are regulated and operate via epigenetic mechanisms and are responsible for the tight

control of Kiss1 expression, mainly in the ARC, during the pubertal transition.

The first element of this counterbalanced system of transcriptional control of Kiss1 that was identified was the PcG of gene silencers, which is composed of 3 major repressor complexes, namely PRC1, PRC2, and PhoRC, which are formed by chromatin proteins and cooperate to silence gene expression [45]. Studies performed in hypothalamic tissue of prepubertal female rats demonstrated that 2 relevant components of PcG, namely EED (which is part of PCR2) and CBX7 (as part of PCR1), interact with the *Kiss1* promoter in the mediobasal hypothalamus (MBH), where the ARC is located. During the early juvenile period, these members of the PcG keep silenced *Kiss1* expression by a repressive histone configuration related to enhanced H3 methylated levels (H3k27me3). At the time of puberty, however, the methylation levels of *Eed* and *Cbx7* promoters increase, thus decreasing their expression; consequently, EED and CBX7 are evicted from the *Kiss1* promoter. As a result, the chromatin is reorganized, from a repressive to a permissive configuration because of the increase of histone modifications, namely H3K9/14ac and H3K4me3, associated with gene activation, which finally results in enhanced *Kiss1* expression [23]; Fig. 2. Very recently, a new PcG-dependent mechanism for the tight control of puberty has been exposed, involving the repressing actions of EED on KDM6B, a histone demethylating enzyme responsible for removing methylation of H3 at the k27 position; H3k27me3 being a repressive mark. Thus, during the infantile period, high EED levels contribute to repress KDM6B, which in turn helps maintain a repressive configuration of H3 upon a set of genes, including *Kiss1*. In contrast, during the pubertal transition, suppression of EED allows the expression of *Kdm6b* and, consequently, elimination of repressive marks leading to activation of *Kiss1* and, therefore, puberty onset [46]. Of note, other factor cooperating with EED in the epigenetic control of *Kiss1* and puberty is SIRT1, which will be reviewed later, as major component for the epigenetic control of puberty by nutritional cues.

However, epigenetic control of Kiss1 neurons and puberty is not held solely by the weakening of the repressive activity of PcG members, but involves also reciprocal changes in members of the TrxG of epigenetic modifiers, which activate gene expression. Thus, in parallel to the decline in EED and CBX7, mixed-lineage leukemia 1 (MLL1) and MLL3, 2 members of TrxG, are abundant at the *Kiss1* promoter during pubertal transition and help define a switch in chromatin configuration, from repressive to active, allowing enhanced *Kiss1* expression; a similar action was found for MLL1 at the *Tac2* (encoding NKB) promoter [47]. In fact, knock-down of MLL1 at the ARC led to reduced *Kiss1* and *Tac2* expression and delayed puberty in female rats [47]. Notably, inactivating mutations of CHD7, another PcG-antagonizing member of the TrxG complex, result in hypothalamic hypogonadism in humans [48]. Altogether, these findings illustrate that an epigenetic switch from transcriptional repression to activation, driven, at least partially, by changes in the balance between PcG and TrxG members at the hypothalamus, and particularly in Kiss1 neurons, plays a master role in the precise timing of female puberty (Fig. 2).

However, PcG is not the only repressor complex acting at central levels and responsible for keeping puberty at check. GATAD1, a member of ZNF family of transcriptional repressors, has been proposed to directly represses human *KISS1* (and *TAC3*) transcription by recruitment of a histone demethylase (KDM1a), which, in turn, reduces the activating histone mark (H3K4me2) at their promoters (Fig. 2). Accordingly, the expression levels of GATAD1 decreased in male and female monkeys during the pubertal transition, and its overexpression in the ARC of immature rats delayed puberty and compromised fertility [49]. Notably, genome-wide studies have documented an association between genetic variation denoted by single nucleotide polymorphisms, located near some ZNF genes, and changes in the age of menarche in women, reinforcing the translational relevance of this epigenetic mechanism of pubertal control [50].

Roles of miRNAs in the central control of puberty

In addition to DNA methylation and histone modification mechanisms, in recent years, a putative role of miRNA regulatory pathways in the control of puberty and the reproductive axis has emerged. Admittedly, regulatory actions of miRNAs upon the HPG axis are known to occur at different levels, including the gonads and the pituitary [51, 52]. Yet, for the sake of consistency, we focus here in reviewing central regulatory mechanisms involving miRNAs.

The first evidence from the potential involvement of miRNAs in the control of puberty came from independent genome-wide association studies that documented that genetic variation near the locus of *Lin28B* were associated to changes in the age of menarche [53-56]. Lin28B is an RNA-binding protein whose major function is to repress the maturation of the large family of miRNAs, let-7, therefore suggesting that miRNA regulatory pathways may participate in the control of puberty. In parallel, studies in mice demonstrated that genetic overexpression of the other member of the Lin28 family, Lin28A, resulted in a delay of puberty onset, further supporting a role of the Lin28/let-7 pathway in pubertal control [57]. In this context, our group has documented reciprocal changes in the hypothalamic expression of Lin28b and members of the let-7 family

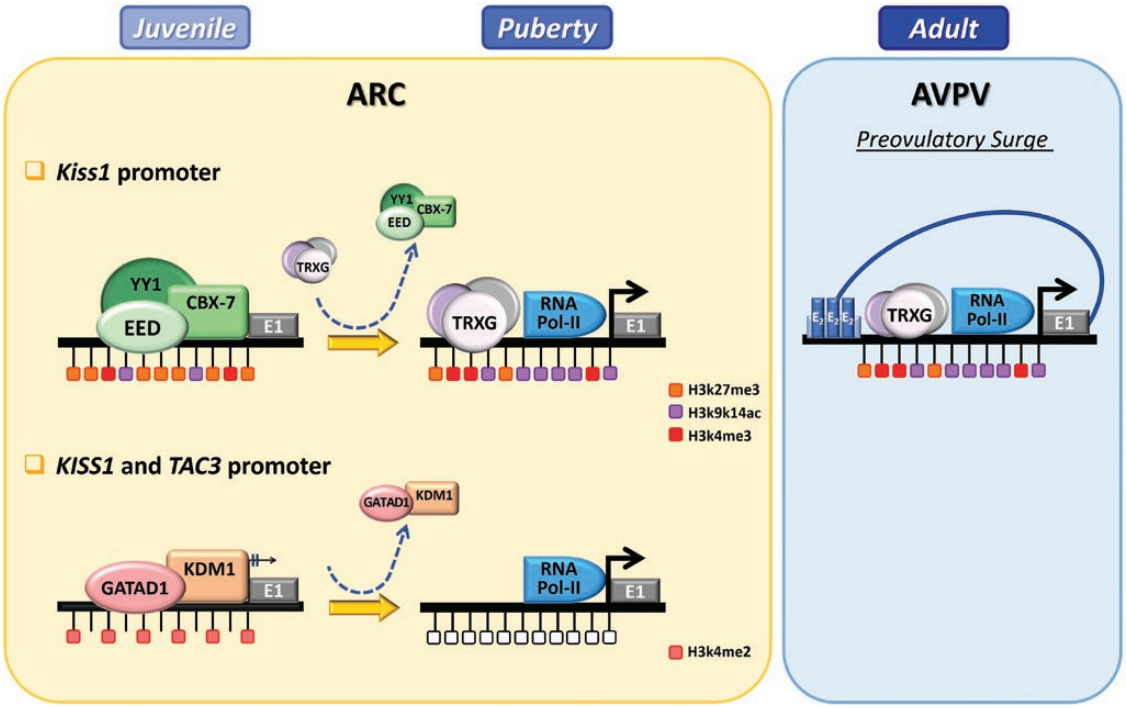

Figure 2. Epigenetic mechanisms for the control of relevant genes in reproduction. A schematic is shown for the major mechanisms described to date in the epigenetic control of key reproductive genes at different developmental periods. Data from rodent studies showed that *Kiss1* expression during pubertal transition is determined by the counterbalance between PcG and TrxG regulatory components, acting at *Kiss1* promoter, in ARC Kiss1 neurons. During the juvenile period, the predominance of PcG elements at its promoter keeps *Kiss1* repressed. During the pubertal transition, the eviction of EED and CBX7 (PcG members), together with the abundance of TrxG elements at *Kiss1* promoter, induces a reorganization of the chromatin landscape. As a result, chromatin changes to a permissive configuration due to the elevation of the activator histone marks, H3K9/14ac and H3K4me3, and the decreased of the inhibitory mark, H3k27me3. Consequently, RNA pPolymerase II (POL-II) is recruited, *Kiss1* expression is activated and puberty is attained. In adulthood, the preovulatory surge of gonadotropins is mediated by an estrogen-dependent activation of AVPV Kiss1 neurons in the female. Thus, the elevation of the circulating levels of estradiol (E2) during the morning of proestrus stimulates acetyl-H3 content (H3K9/14) at *Kiss1* promoter, leading to AVPV-specific *Kiss1* expression upregulation. Finally, it has been recently documented the implication of ZNF proteins in the control of *KISS1* and *TAC3* expression. At the infantile period, GATAD1, a ZNG finger protein, recruits a histone demethylase, KDM1A, to *KISS1* and *TAC3* promoters, leading to demethylation of the activator mark H3K4me2 and therefore blockade of *KISS1* and *TAC3* expression. During pubertal transition, eviction of the repressor complex (GATAD1/ KDM1A) permits enhanced *KISS1* and *TAC3* expression and pubertal activation002E.

during the postnatal/pubertal transition, with a significant decline in Lin28B mRNA, associated with increased expression of let7 members as well as the related miRNAs, mir-132 and miR145 [58]. In addition, experimental manipulations altering brain sex differentiation and/or puberty altered the expression ratios of Lin28/let-7 at the time of puberty [58], which further suggests that hypothalamic Lin28/let-7 system is involved in the developmental mechanisms leading to puberty onset.

In addition, a role for miRNAs regulatory pathways acting in GnRH neurons in the control of puberty onset has been proposed based on functional genomic studies and a combination of expression and mechanistic analyses. First, mice engineered to lack Dicer, the enzyme responsible for the final maturation steps in the synthesis of miRNAs selectively in GnRH neurons, displayed absence of complete pubertal maturation and infertility of central origin, suggesting a master role of miRNAs in the whole activation of GnRH neurons that takes place at puberty [59]. This contention was further consolidated by functional data supporting a key role of miR-200/-429 and miR-155 in the control of puberty, acting at hypothalamic GnRH neurons. In detail, miR-200/-429 and miR-155 in GnRH neurons can suppress the expression of ZEB1 and CEBPB, respectively; both are repressors of GnRH neurosecretion through direct and indirect mechanisms. Hence, the increase of expression of miR-200/-429 and miR-155 in GnRH neurons that takes place in GnRH neurons during the infantile/juvenile transition inhibits this repressive system that keeps GnRH secretion low before puberty, and therefore plays a fundamental role in enhancing GnRH promotor activity at early prepubertal periods [59].

More recently, our group has documented a putative role of miR-30b in pubertal control because of its capacity to repress Mkrn3 in the hypothalamus. Mkrn3 is a maternally imprinted gene encoding the makorin RING-finger

protein-3, whose deletion causes central precocious puberty in humans [60]. Consistent with a potential role as pubertal repressor, the hypothalamic expression of Mkn3 is reduced before puberty in rodents [60, 61]. Notably, the 3′-UTR of Mkrn3 is highly conserved, indirectly suggesting putative miRNA-mediated regulatory mechanisms, acting at this region. In fact, by a combination of bioinformatic and functional studies, our group has documented that miR-30b, whose hypothalamic expression increases postnatally, operates as a repressor of Mkn3 to control puberty onset, acting via 3 binding sites in a highly conserved region of its 3′-UTR [61]. Thus, miR-30 would act as a repressor of a repressor, whose activation may contribute to pubertal onset. Of note, our study preliminarily documented the expression of miR-30 and Mkrn3 in Kiss1 neurons [61]. This is compatible with a recent study supporting that the inhibitory actions of Mkrn3 on puberty onset are conducted at Kiss1 neurons [62]. The actual role of miRNAs in Kiss1 neurons in pubertal control needs to be further studied, but our initial data demonstrate that Dicer ablation in Kiss1 neurons perturbs puberty (mainly female) and causes central hypogonadism in mice (*our unpublished data*).

Epigenetic Regulation of Adult Reproductive Function

The contribution of epigenetic regulatory mechanisms in the central control of adult reproductive function has also been explored. A key feature of female reproduction is the cyclic generation of the hormonal drive of ovulation, the so-called preovulatory surge of gonadotropins, which occurs only in females. In rodents, this surge is generated by the episodic activation of Kiss1 neurons at the AVPV, where estrogen, whose levels rise in the morning of proestrus (ie, the few hours before the preovulatory surge), exerts a potent positive feedback action, leading to enhanced expression of Kiss1. Intriguingly, this positive feedback at the AVPV is opposite to the negative feedback action of sex steroids at ARC Kiss1 neurons, but the molecular basis for such dichotomy has not been fully clarified. In this context, Tomikawa et al reported that elevated circulating estradiol levels, preceding the preovulatory surge, induces opposite changes in histone acetylation, but not in DNA methylation, at the Kiss1 promoter of AVPV- vs ARC-kisspeptin neurons. In the AVPV, estradiol stimulated acetyl-H3 content (H3K9/14), leading to AVPV-specific Kiss1 upregulation at proestrus [63]. In contrast, it induced H3 deacetylation in the ARC, decreasing Kiss1 expression. Notably, these epigenetic regulations caused by estradiol occur repeatedly to switch on and off the same promoter regions in different hypothalamic areas. In addition, estradiol positive feedback involves recruitment of estrogen receptor (ER)α to the Kiss1 promoter and changes in chromatin looping, exclusively in AVPV neurons, where an estrogen-responsive enhancer intergenic region, located downstream of Kiss1, was described [63]. Altogether, this evidence suggests that differential histone modifications, involving acetylation/deacetylation, in the populations of Kiss1 neurons play a major role in the cyclic control of ovulation.

Notwithstanding this information, DNA methylation changes have been also associated to the regulation of Kiss1 expression in adulthood. For instance, the presence of higher ERα and Kiss1 expression in the preoptic area (holding the AVPV) of female than in male mice in adulthood was correlated with steroid-mediated changes in the methylation levels of their promoters [22]. However, contrary to the expected function of methylation as suppressor of gene transcription, the Kiss1 promoter was hypermethylated in females, suggesting a potential interaction between DNA methylation and inhibition of transcriptional repressors of Kiss1 [22].

Environmental Control of Reproduction Via Epigenetic Mechanisms: Nutritional Cues and Endocrine Disruptors

Reproductive function in general, and puberty in particular, are extremely sensitive to multiple environmental signals, from nutritional cues to manufactured and natural compounds with capacity to act as endocrine disruptors (EDCs) [11, 64, 65]. Epigenetic mechanisms are very well suited for transmitting the modulatory influence of such environmental signals, via mechanisms that are yet to be fully disclosed. In this section, we briefly recapitulate what is known about the epigenetic influence of the nutritional status and EDCs on puberty and reproductive function, with major focus on central mechanisms.

Nutrition and the Epigenetic Control of Reproduction

The nutritional status is a major modulator of virtually all bodily functions, including pubertal maturation and fertility. Changes in energy status are specially important during critical developmental windows [66], occurring in utero or during early postnatal periods, when alterations of nutrient and/or energy availability may induce permanent changes in the developmental program of different homeostatic systems, resulting in persistent perturbations in the individuals or their offspring and leading to increased vulnerability to health perturbations later in life, such as diabetes, breast cancer, and cardiovascular and metabolic diseases. Early nutritional programing has also discernible

impact on pubertal timing and adult reproductive function, as illustrated by preclinical studies [67-69]. Because nutrition is a direct source of methyl and acetyl groups, changes in dietary habits, especially during gestation or early developmental periods, can have an important impact in key epigenetic regulatory mechanisms, such as DNA methylation and histone acetylation, with durable consequences during the lifespan [70, 71]. Yet, the precise epigenetic mechanisms whereby early nutritional changes affect later reproductive maturation and function remain unfolded.

In addition, puberty and fertility are sensitive to actual changes in nutritional cues and energy reserves (ie, those contemporary to pubertal maturation or in adulthood) that have also a profound influence on different reproductive traits [11]. Reproduction is an energy-demanding process, especially in females, and therefore the acquisition and preservation of reproductive capacity is subordinated to energy reserve levels. Accordingly, conditions of persistent metabolic stress, especially those linked to energy deficit, are commonly bound to alterations in the timing of puberty and fertility, whereas a threshold of body fat mass is required to achieve reproductive capacity [72]. Such metabolic control of the HPG axis is largely conducted by a number of hormones, such as leptin, insulin, and ghrelin, capable of modulating the reproductive axis as a function of the metabolic status of the organism [11, 72]. For instance, conditions of energy deficit, signaled by low leptin and high ghrelin levels, results in suppressed HPG axis function at central levels.

Although the precise molecular mechanisms by which the information conveyed by this plethora of metabolic hormones is transmitted to the brain centers governing the reproductive axis are not completely elucidated, experimental studies conducted by our group in recent years have focused on addressing the potential role of cellular energy sensors, such as the mammalian target of rapamycin and the AMP-activated protein kinase, in the central control of the HPG axis, and, in particular, the modulation of Kiss1 neurons [73-75]. In this context, our recent studies have documented a novel epigenetic mechanism involving SIRT1 activity in Kiss1 neurons, with a key role in the metabolic control of puberty. In mammals, the family of Sirtuins consists of 7 members SIRT1-7, of which SIRT1 is the most evolutionarily conserved. SIRT1 is a NAD^+-dependent class III deacetylase acting on a wide range of targets, as histones and p53, with pleiotropic regulatory actions in numerous biological processes [76, 77]. SIRT1 operates as a cell energy sensor, whose activation is dependent on an increased NAD^+/NADH ratio, which occurs in conditions of energy deficit [76]. Although Sirtuins operate in multiple tissues, part of their actions are conducted in the brain, including the hypothalamus, as denoted by the fact that Sirt1 expression is detected in ARC and increases moderately after fasting [78], whereas brain-specific SIRT1 overexpression increases lifespan in mice [79].

Although a potential involvement of Sirt1 in the central control of reproduction had been previously suggested [80, 81] putatively via control of GnRH migration, our recent findings in female rodents were the first to document a role of SIRT1, operating in ARC Kiss1 neurons, as a major epigenetic regulator of puberty, and its modulation by nutritional cues and energy status. Notably, SIRT1 protein levels in the MBH, as determined by Western blot, decreased during the infantile-to-pubertal transition and in models of advanced puberty because of early-onset obesity, whereas undernutrition caused an elevation of hypothalamic SIRT1 protein content [82]. These changes were opposite to those of *Kiss1* expression, and functional activation of SIRT1 by different approaches (pharmacological or genetic; the latter, global or ARC-specific overexpression) was related to the decline of *Kiss1* expression in MBH and delayed puberty, suggesting SIRT1 is a repressor of *Kiss1*. This contention was confirmed by chromatin-immunoprecipitation assays, which delineated the epigenetic mechanism through which SIRT1 regulates *Kiss1* expression, mainly at the ARC, in a nutrient-dependent manner [82]. During postnatal maturation, premature initiation of puberty is prevented by SIRT1 recruitment of the PcG member, EED, to *Kiss1* promoter, which leads to a repressive chromatin configuration that keeps *Kiss1* expression inhibited. When pubertal timing is approaching, SIRT1 is removed from the *Kiss1* promoter, the chromatin changes to an active state, based on the elevation of activating histone marks (H3K9/16ac), together with the reduction of repressive marks (H3K4me), and this change in the chromatin landscape allows the increase of *Kiss1* expression and thereby initiation of puberty [82]. Chronic undernutrition leads to a delay in such developmental switch, with protracted SIRT1-mediated repression of *Kiss1* expression. In contrast, early-onset obesity causes a decrease of SIRT1 content in Kiss1 neurons, which accelerates the eviction of SIRT1 from the *Kiss1* promoter, therefore enhancing *Kiss1* expression and advancing puberty [82]. Our as yet unpublished data suggest that SIRT1 may also operate at AVPV Kiss1 neurons to repress *Kiss1* expression and participate in the nutritional control of the preovulatory surge in adulthood.

EDC and the Epigenetic Control of Reproduction

Reproductive maturation and function can be influenced also by a wide range of both natural and synthetic compounds with capacity to mimic, block, or interfere with multiple endocrine pathways, globally termed EDCs. These include multiple compounds commonly used in everyday

life, such as perfumes, plastic food containers, pesticides, metals, and phytoestrogens. The concern about the adverse effects of exposure to EDCs, and subsequent studies to search for the molecular mechanisms underlying their impact on reproduction, has grown over the past decades. Notwithstanding, interpretation of the pathophysiological mechanisms of EDC actions remains complex because EDCs operate following different routes of absorption via multiple pathways and at different temporal windows. Moreover, possible additive or even synergistic effects between different coexisting EDCs can occur. All of these features have urged in-depth research on the reproductive impact of EDC.

Despite the multiplicity of mechanisms whereby EDC can influence the reproductive axis, alteration of the epigenetic regulatory machinery is considered one of the most prominent modes of action whereby EDC exert their long-term effects [83]. Among the multiple EDCs, those able to act via and/or modulate estrogen or androgen signaling are possibly the most relevant in terms of reproductive impact. A large body of evidence indicates that early developmental exposure to such EDCs can affect the maturation and adult function of the reproductive system in animals and humans [84]. Reproductive effects of ECDs in mammals likely include, but are not limited to, suppressed gametogenesis in both male and female, decreased semen quality, genital malformations, premature ovarian failure, endometriosis, polycystic ovary syndrome, alterations in brain masculinization or feminization, pubertal disorders, and perturbed sex and social behavior, among others.

Although extensive recapitulation of the reproductive effects and mechanisms of action of all EDC is beyond the scope of this review, for illustrative purposes, we briefly summarize here the impact of bisphenol A (BPA) on the central components of the HPG axis, and putative epigenetic mechanisms involved. BPA is considered one of the most abundant EDC, with solid evidence on high exposure levels and strong estrogenic activity [85], and, therefore, potentially involved in a wide range of health problems, including reproductive disorders. In this context, the potential impact of BPA exposure in puberty has been addressed by numerous epidemiological and preclinical studies. However, the results have been elusive or even contradictory, with discordant results depending on dose, route, or timing of administration [86-90]. For instance, the link between increased urinary levels of BPA and delayed puberty in girls was reported in some studies, but not others. In the same vein, postnatal exposure to very low doses of BPA delayed puberty in rats, whereas high doses evoked the opposite effect [91]. Our group has recently demonstrated that perinatal exposure to environmentally relevant doses of BPA substantially advanced external signs of puberty onset in female mice, but consistently suppressed circulating LH levels [92]. In addition, such BPA exposure modulated the development of different populations of Kiss1 neurons, with an elevation of the number of kisspeptin-positive fibers and neuronal cell bodies in AVPV, whereas in the ARC, low-dose BPA exposure induced a decrease of kisspeptin content. More relevant, although BPA treatment did not block the well-characterized increase of *Kiss1* and *Tac2* expression at the ARC during pubertal transition, the levels achieved were significantly reduced compared with control animals. In sum, our data illustrate the impact of low doses of BPA at a critical developmental window in the timing of puberty, inducing a potent suppressing effect on ARC Kiss1 neurons and LH secretion in pubertal female mice.

The specific molecular mechanisms through which BPA affects the neuroendocrine control of puberty remain poorly understood. However, epigenetics might be involved because exposure to BPA has been shown to induce epigenetic alterations (eg, changes in methylation or histone modifications) in the genome of humans and mice [93, 94], as well as changes in the expression and activity of miRNAs [95]. Part of these epigenetic mechanisms take place centrally. For instance, gestational BPA exposure has been found to alter DNMT1 and DNMT3A levels in the cortex and hypothalamus of the juvenile offspring, leading to an abnormal estrogen receptors expression and sexual behaviors [96]. In same the vein, neonatal administration of BPA induced hypermethylation of the promoters of ERα and ERβ in rat testis, affecting to spermatogenesis and male fertility [97], suggesting that the capacity of BPA to alter DNA methylation manifest at different levels of the HPG axis. BPA also increased the activity of histone acetyltransferases, resulting in increased levels of histone acetylation in zebrafish embryos and spermatozoa [98]. Despite the known central effects of BPA, targeting key neuronal populations of the reproductive brain, such as GnRH and Kiss1, whether this compound alters epigenetic regulatory mechanisms in such neurons (and if so, which ones) remains largely unexplored and warrants future investigation. Initial evidence suggests that BPA is able to induce a shift in the subcellular location of Tet2 in GnRH neurons and changes in H3 methylation at the GnRH promoter in mice [44].

Conclusions and Future Directions

Compelling evidence, gathered in recent years, has documented the important role of epigenetic regulatory mechanisms in the control of different aspect of development and function of the HPG axis, from sex differentiation to puberty and adult fertility. Epigenetics provides another layer of sophistication in the complex mechanisms whereby sexual maturity is achieved and fecundity is attained and

preserved in mammals, including humans. Although much has been learned regarding the different epigenetic mechanisms that operate in concert to physiologically control the reproductive axis, using mainly rodent studies, less is known about the actual contribution of deregulated epigenetic pathways as pathophysiological substrate for common reproductive disorders. Yet, clinical and preclinical evidence strongly suggests this is a tenable possibility for a wide spectrum of conditions, ranging from pubertal alterations bound to nutritional disorders to polycystic ovary syndrome (PCOS). Importantly, deregulated epigenetic marks may not only be important from a pathogenic perspective, but may also serve as biomarkers and even targets of intervention for such reproductive diseases. As a prominent example, a recent study has documented that changes in DNA methylation patterns (mainly, hypomethylation) are found in a preclinical model of PCOS, but also in women with this condition [99]. Moreover, treatment of a mouse PCOS model with the methyl donor, S-adenosyl methionine, was capable to reverse its major metabolic and neuroendocrine alterations [99], suggesting this may be a target for treatment of this prevalent disease. Likewise, changes in circulating miRNAs have been reported in patients with PCOS, which may contribute to the alterations of the syndrome buy may also help to define novel diagnostic tools for improved classification of PCOS women [100]. Although characterization of some of the facets of these complex regulatory phenomena is still rather incipient, these features illustrate the exciting avenues that epigenetics offers for a better understanding, diagnosis, and eventual treatment of highly prevalent reproductive disorders in the near future.

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Additional Information

Correspondence: Maria Jesús Vázquez, Department of Cell Biology, Physiology & Immunology, Faculty of Medicine, University of Córdoba, Avda. Menéndez Pidal s/n. 14004 Córdoba, Spain. E-mail: bc2vavim@uco.es; or Manuel Tena-Sempere, Department of Cell Biology, Physiology & Immunology, Faculty of Medicine, University of Córdoba, Avda. Menéndez Pidal s/n. 14004 Córdoba, Spain. E-mail: fi1tesem@uco.es.

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