Unravelling the role of epigenetics in reproductive adaptations to early-life environment

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Key Points

- Human reproductive function adjusts to changing environmental conditions
- Key 'windows of susceptibility' during various stages of early development are the most sensitive to events or exposures which can impart long-term re-programming of adult reproductive function
- Epigenetic modifications have a role in regulating the central control of reproduction and pubertal onset, and likely mediate much of the adaptive response
- Human cohort data is useful for identifying methylation in proxy tissues that correlates with phenotypic variation, but determining cause-effect is challenging because hormones affect the epigenome and epigenetic ageing
- Understanding which of the modifications are functional and responsible for the phenotype requires integrating the study of human tissues, animal and cell models and molecular approaches
- Characterization and elucidation of these adaptive mechanisms are needed to inform the clinician of alternative reproductive strategies, and the implications for fertility treatment and healthy ageing

Abstract

Reproductive function adjusts in response to environmental conditions in order to optimize success. In humans, this plasticity includes age of pubertal onset, hormone levels and age at menopause, all of which have broad health implications. These reproductive characteristics vary across populations with distinct lifestyles and following specific childhood events, and point to a role for the early-life environment in shaping adult reproductive trajectories, as well as moderation of the response by other biological systems. Epigenetic mechanisms respond to external signals, exert long-term effects on gene expression, and have been shown in animal and cellular studies to regulate normal reproductive function, strongly implicating their role in these adaptations. Moreover, human cohort data have revealed differential DNA methylation signatures in proxy tissues that are associated with reproductive phenotypic variation, although the cause-effect relationships are difficult to discern, calling for additional complementary approaches to establish functionality. The influence of childhood environment on adult reproductive function, with associated health consequences, is an important consideration in understanding how reproduction is regulated, and necessitates consideration by clinicians treating women with diverse life histories. Resolution of the molecular mechanisms responsible for human reproductive plasticity could also lead to new approaches for intervention by targeting these epigenetic modifications.

[H1] Introduction

The reproductive endocrine axis is dynamic and responds to multiple environmental signals that provide the plasticity for optimization of reproductive success over an individual's life-span[1,2] . The mechanisms governing and regulating this adaptability are not fully understood but changes in the regulatory epigenetic landscape (**Box 1**) undoubtedly play a central role. The epigenome is responsive to external signals and is able to modify gene expression patterns and networks[3], enabling it to direct changes in reproductive function in response to differing environments. However, the effects of early-life circumstances on adult reproductive function vary, depending both on the timing of events relative to 'windows of susceptibility', and between individuals growing up in industrialized settings and those in the developing world who have very different life styles[4–9]. This plasticity and the precise nature of the altered reproductive traits likely depend on resource availability, and clearly involve interactions between multiple physiological systems[10–13].

The role of the epigenome in the central control of reproduction has been the focus of several studies utilizing animal and cell models over the past decade or so[14–28], whose findings comprise the basis of our current understanding on how adaptive reproductive strategies can be implemented. However, a reciprocal interaction also exists whereby reproductive hormones affect the epigenome and epigenetic ageing[29–34], which complicates distinguishing cause from effect. This ambiguity is especially important when trying to understand regulatory processes from human data, in which experimental work and tissue accessibility are limited. Consequently, the importance of correlations identified in many of 'big data' sets from large human population cohort studies is often unclear, and the mechanistic basis of particular phenotypes remains ambiguous. More integrated approaches are warranted, including animal models to verify that the epigenetic modifications occur in the functional tissues. Subsequently, manipulations and *in vitro* experiments are necessary to define the actual role of the modifications on gene expression and whether they are responsible for the phenotype.

While adaptations to the environment can be seen as beneficial for the individual, they carry health consequences that are often far-reaching. The timing of puberty, circulating hormone levels and reproductive life span all affect cancer predisposition, while age at menopause is not only consequential for women who postpone pregnancy until later life, but is also associated with the onset of osteoporosis and age-related mortality[1]. Furthermore, in the clinic, diagnostic standards are typically derived from data of women in industrial, northern and/or western countries, where levels of circulating hormone are characteristically higher than in women living in other settings[35]. Increasing numbers of women are migrating around the globe and seeking medical care from doctors who are often only familiar with normative standards from these restricted populations. It is therefore imperative to characterise these adaptive responses and elucidate the mechanisms responsible, so that the clinicians can make more informed decisions. Clarification of some of these mechanisms might also open the way for developing novel clinical approaches to adjust the reproductive phenotype by targeting the epigenome.

In this review, we address the enigma of how adult reproductive function can be shaped by childhood events. We also examine the evidence for the epigenetic regulation of key regulatory genes that govern the central control of reproduction and thereby emerge as pivotal elements in the adaptive response. We discuss this conceptual approach in understanding how variation in mammalian reproduction is regulated epigenetically, and emphasize the need for multifaceted approaches to elucidate this intriguing aspect of human biology.

[H1] Plasticity of human reproduction

[H2] Reproductive phenotypes adapt to the environment

The human reproductive phenotype seems to be well programmed, with sexual maturation and menopause typically occurring within predictable age ranges in a given population[36,37]. The constraints on the timing of these events remain largely a mystery, but the central control of reproduction involves a complex interplay between numerous physiological systems which, when challenged, reveals considerable plasticity and adaptation to environmental conditions.

Changes in reproductive function comprise both rapid responses to immediate challenges (such as physiological, metabolic or psychological stress) and long-term adaptation to the environment in order to optimize individual survival and reproduction under diverse conditions[1,38]. This plasticity ensures that, during times of abundance, investment can be made in multiple physiological systems including growth, maintenance, and reproduction. However, in the face of harsh conditions and deprivation (e.g. infection or nutritional deficit), fewer resources can be allocated to reproduction, which might even be shut down[39,40]. Life history theory describes and rationalizes such trade-offs in varying environmental conditions where the costs of immediate and even future reproductive activity are assessed against the need to invest in individual maintenance and survival[41–43]. (**Fig 1**).

In accordance with the prevailing environmental conditions, age at normal pubertal onset varies across populations and between 1890-1990 advanced by as much as 3 y in industrialized countries[44]. Many of these changes are associated with nutritional status, improved health and increased growth rates, although some studies have highlighted differences between distinct ethnic groups[45–54]. However, the role of environment, as distinct from genetics, was shown in a series of studies in male and female Bangladeshis, some of whom migrated to the UK. In these studies, age at puberty was later in children who had grown up in Bangladesh compared to those who had migrated to the UK as young children or were second generation migrants; the latter two groups entered puberty at a similar age to their European-ethnic neighbours[7,55,56]. Other studies have also reported shifts in age of pubertal onset in migrant populations, some of which have been attributed to 'catch-up growth', although the advancement of puberty following migration is apparent also in children who do not come from deprived backgrounds[6,46,57].

Adult reproductive function also varies between individuals who grow up in different environments (**Table 1**). Lower average reproductive hormone levels are consistently reported in populations who live in more energetically-challenging environments[58–65]. In people with Bangladeshi heritage who were born in the UK or migrated there as young children, progesterone levels in women and testosterone levels in men are markedly higher than those of individuals who grew up in Bangladesh, and similar to those of UK-born ethnic Europeans[6,7]. Despite progesterone levels that would be considered insufficient in Western clinics, individuals in non-industrialized societies are fertile, suggesting distinct programming to allow reproductive function within these lower hormonal ranges[35,61,66].

Compared with women who grow up in developed countries, women who grow up in less developed countries characteristically experience menopause at a younger age, in addition to having a later puberty, and this is also dependent on childhood environment[5,56,67,68]. The longer reproductive life span and elevated hormone levels seen in Bangladeshi child migrants to the UK were not observed in women who migrated as adults, even after spending a considerable number of years in the UK, indicating that the childhood environment is the main determinant of this adult phenotype[5,6].

[H2] Responses to early-life adversity vary

Although changes to the early-life environment can induce adaptive responses in pubertal timing and adult reproductive function, the actual consequences of exposure to adverse events can be puzzlingly diverse[4,8,13]. It should be noted, however, that most scientific studies are carried out in western, educated, industrialized, rich and democratic populations, in which lifestyles and nutrition are far

removed from those of our ancestral environments as hunter-gatherers, and in extant foraging societies[4,69]. In fact, differing responses are seen in populations with distinct cultures. For example, in developed countries, girls who experience paternal absence during childhood are widely reported to have an earlier puberty relative to their peers, while in less developed countries, girls exposed to similar events show either no change or even delay in pubertal onset[4,70,71]. These findings suggest that the distinct outcomes are due to differences in the physiological or metabolic states in these girls, which are determined by their environment.

Physiological responses to adversity imposed by harsh conditions, are usually energetically costly, as exemplified during inflammation or infection by the proliferation of immune cells. In states of limited resources, trade-offs are required between these physiological systems as described in life history theory[12,72,73]. In fact, chronic inflammation during childhood is often associated with pubertal delay[74,75], while an increased level of body fat in Amazonian forager children was seen to endow them with protection from growth-inhibiting effects of acute inflammation[73]. Thus, the specific outcome of early-life adversity on reproductive function almost certainly varies according to the availability and allocation of resources. (**Fig 1**).

The hypothalamic–pituitary–adrenal (HPA) axis has a key role in allocation of metabolic resources through the centralized 'stress response', which affects reproduction as well as growth and homeostasis. Increased stress and elevated cortisol are usually associated with reduced reproductive function and delayed puberty[76–78]; however, the stress response also seems to mediate the effects of poor nutrition on the timing of pubertal onset, as seen in stressed rats fed a Western diet (high levels of refined sugars and saturated fats, and low fiber content) in which normal pubertal timing was restored following environmental enrichment[11]. Moreover, the set-point of the HPA axis can be changed following early-life trauma, resulting in chronically altered circulating cortisol levels, while stress responses are attenuated in some metabolic states, such as conditions of low visceral adipose tissue[79–82]. It is clear, therefore, that understanding the mechanisms behind adaptive reproductive strategies needs to take into account the complexities of the wide-ranging physiological context.

[H2] Population studies, big data and finding the role of epigenetic programming

One of the ways to overcome the difficulties faced in analyzing data with large variation is by looking at extensive cohort studies of human populations. This approach has revealed altered reproductive phenotypes that correlate with stressful conditions amongst diverse populations[83–88]. Many of these databanks maintain records of circulating hormone levels, which can inform about reproductive competence and state of the ovarian reserve, and some also have blood or buccal DNA methylation data that might relate to the phenotypic variation. Such connections, however, are problematic given that methylation patterns differ across tissues[89,90], providing an ambiguous link between any changes observed in these proxy tissues, which do not express most of the key factors that regulate reproductive function, and epigenetic modifications present in the functional tissues[89,90]. Therefore, understanding the underlying mechanisms behind these observations remains challenging, both due to inaccessibility of relevant tissues, and also because cause and effect are very difficult to discern from longitudinal datasets.

Epigenetic modifications have been proposed to underlie altered adult phenotypes in response to early-life events, as described in the context of Developmental Origins of Health and Disease (DOHaD)[91,92]. However, evidence to date for such modifications in the context of the central control of reproduction remains mostly indirect, in contrast with the metabolic and stress axes for which human and animal studies have demonstrated more fully a role for epigenetic modifications in adaptive responses to external signals[93–95]. Glucocorticoids play a central role in the modified stress response as demonstrated by the epigenetic re-programming of genes in the HPA axis, including the glucocorticoid receptor[95,96]. Furthermore, glucocorticoid catabolism is decreased through reduction

in the steroidogenic enzyme 5α reductase-1 in the liver, which was proposed to increase levels of active cortisol in order to enhance fuel output[80].

We have found that a *cis*-regulatory region of *SRD5A1*, the gene that encodes 5α reductase-1, is more methylated in buccal DNA of Bangladeshi women who had grown up in Bangladesh rather than in the UK. This gene was also more methylated and its expression down-regulated in a mouse model of early-life adversity that we used to study the epigenetic basis for the women's adaptive reproductive phenotype (B.B.-S., L.P., Kurshida Begum [K.B.], Gregory Leeman [G.L.], Richard D. Emes [R.D.E.], R.S., G.R.B., P.M. unpublished work). In the hypothalamus, this enzyme moderates activity of the HPA axis as well the hypothalamic-pituitary-gonadal (HPG) axis through the production of neurosteroids[97–99] , so appears to function as an epigenetically-regulated sensor that can determine the appropriate resource allocation to each of these axes in challenging environments.

[H1] Windows of susceptibility

[H2] DOHaD and adaptive programming during fetal development

The concept of DOHaD is firmly rooted in the gestational period, having arisen from David Barker's observations of differences in birth weight across England, and the associated later health outcomes[100]. Accordingly, most studies on the effects of early-life events on later health and disease have focused on very early stages of development, during which the patterns of gene expression that direct differentiation and organogenesis first become established. Barker and others have described fetal metabolic programming, as determined by the nutritional state during gestation, as shaping the trajectory of resource allocation and thus also health throughout later life[93,101–103]. At least some of these effects were seen to occur independently of the uterine environment, strongly supporting an epigenetic component[102–104].

Maternal nutrition has been shown repeatedly to affect DNA methylation patterns in the offspring of various mammals[105]. Notably, the level of methyl donors in the maternal diet at conception was seen to alter methylation levels at several metastable epialleles in the children of women from rural Gambia[106]. In mice, maternal nutrition was noted to influence methylation in the off-spring at several imprinted genes, as well as *leptin* and *Ppara* and a number of genes encoding chromatin modifiers[9,107–110]. Changes in the epigenetic landscape, most notable at genes like these that encode factors regulating growth and metabolism, have been proposed to affect the offspring's metabolic phenotype as part of an adaptive response to the early-life adversity[111]. This adaptive response results in altered birth weight and childhood growth rates, nutritional state and metabolic rates all of which have key roles in pubertal timing, as seen in the early pubertal onset of low birth weight children that undergo "catch-up" growth[112–114].

Poor maternal nutrition and low birth weight of female offspring are also associated with a decrease in the size of ovarian reserve, which is evident in earlier reproductive senescence in women and rats [115–120]. The pool of germ cells is established during gestation, and in the female reaches a finite number, such that events during this time can have a marked effect on female reproductive function later in life. The effect of the uterine environment on the offspring's ovarian development is seen in neonatal rats born to nutritionally-deprived mothers, which contain more activated primordial follicles and altered levels of PI3K–Akt signaling (which regulates follicle recruitment)[121].

Further to these data, after pubertal onset, reduced rates of ovulation and disrupted estrous cyclicity are reported in many women who had low birth weights and those born to nutritionally-deprived mothers, and this is also observed in mice[121–123]. The fact that infants born small for gestational age have elevated circulating FSH levels indicates that the central control of reproduction is also modified

in these individuals[124]. Indeed, the neuroendocrine regulation of reproduction is being established and primed during fetal development, including the sexual dimorphic masculinization of the male brain by fetal-origin androgens, further contributing to the sensitivity of the fetal period to epigenetic perturbations[125].

[H2] The neonatal period and 'mini-puberty'

The hypothalamus and pituitary undergo considerable postnatal maturation to establish the central mechanisms that regulate reproduction in the adult[126–128]. Changes in epigenetic modifications during the key developmental stages thus continue to have a role in adaptive reprogramming in response to the neonatal and childhood environment, possibly via distinct mechanisms depending on the age of exposure and fluctuations in growth and metabolism that characterize these stages. (**Fig 2**).

During the 'mini-puberty', which occurs in the first few months of life, activity of the hypothalamic–pituitary axis is stimulated due to the immediate postnatal drop in steroid negative feedback[127,129]. In boys and male rodents, this activity includes a very rapid and transient testosterone surge on the day of birth[125,130–132]. The hormonal changes during this time modify hypothalamic neuron growth and development, gene expression patterns and the epigenome, and the androgen exposure leads to unique characteristics associated with brain masculinization (starting during the latter part of fetal development). Therefore, this neonatal period comprises a clearly defined window which is, at least in part, sexually-dimorphic[125,132].

The process of hypothalamic maturation during this time is acutely sensitive to nutritional state, particularly the kisspeptin neurons in the arcuate nucleus that regulate gonadotropin releasing hormone (GnRH) synthesis and secretion. The development of these cells is adversely affected by poor nutrition, which is reflected in reduced neuron density, reduced *Kiss1* and GnRH expression and changes in age of pubertal onset and fertility[133–135]. Moreover, the testosterone surge in these first few postnatal months was reported to correlate with growth and metabolism in early childhood[136,137], which likely affects pubertal timing.

The gonadotrope population of the pituitary also undergoes dynamic change in the neonatal period, with a transient wave of expansion of precursor cells that undergo proliferation and differentiation. In mice, this includes epigenetic re-programming, some of which involves a major drop in the expression of ten–eleven translocation methylcytosine dioxygenase1 (TET1) DNA hydroxymethylase[23]. Subsequently, however, the numbers of gonadotropes are reduced to adult levels[138,139] and the function of this temporary increase in hypothalamic–pituitary activity on later reproductive function is not clear. It is reasonable to assume, however, that this developmental phase also has a role in programming the central control of reproduction in the adult.

[H1] Sensitive windows during childhood

Early childhood is dominated by changes in growth and metabolism rather than reproductive activity, which is largely quiescent until the approach of puberty. However, numerous studies have pointed to early or mid-childhood as being a critical time for mediating the effects of adversity on reproductive function[5–7,140–142]. One reason for sensitivity at this time is the changing levels of hormones involved in somatic growth and bone maturation, which have a role in determining the timing of pubertal onset[143]. As noted, factors that regulate metabolic function seem particularly sensitive to epigenetic modification and changes in childhood growth rates or metabolic status certainly influence the timing of pubertal onset [126,144]. Levels of body fat also vary considerably during this time, especially in girls[145], which affects the trade-offs between homeostasis, growth and reproduction that occur in adverse conditions (**Fig 1**). Differences in the size and quality of these metabolic reserves might also explain some of the diverse responses in distinct populations.

At around 5-8 years of age, children undergo adrenarche, signalling maturation of the HPA axis and a dramatic increase in circulating levels of dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS). These 'weak' androgens (i.e. they bind the androgen receptor with low affinity) can be converted to stronger androgens or estradiol in specific tissues, including the brain, gonads, liver and adipose tissue[146], which allows them to bind the steroid receptors and presumably trigger epigenetic modifications. DHEA and DHEAS exert a multitude of actions, including modifying neurotransmitter receptors in the central nervous system, modifying the stress and immune responses and affecting bone density and metabolism[146], any of which might affect pubertal timing and/or the central regulation of reproduction. In adults, they also act on the ovary directly, increasing circulating Anti-Müllerian hormone (AMH) levels as well as the ovarian response to stimulation, suggesting their role in ovarian follicle recruitment[147,148]. The broad effects of these androgens, including their direct effects on the HPG axis, corroborate the likelihood that early-to-mid childhood comprises a major developmental milestone in reproductive programming. This observation is supported by studies in the Bangladeshi migrants, in which 8 years of age was found to be a critical 'cut-off' age in girls and boys that influenced the adult reproductive phenotype[5–7].

Most studies suggest that early-life is the principle phase during which adaptive reprogramming occurs. Changes in the epigenome, however, continue throughout the lifespan, both during normal developmental processes and in response to specific environmental signals, and are pivotal in the onset of puberty. The epigenome then continues to mediate the central control of reproductive function, sustaining the reproductive trajectory established by the early-life programming, while also endowing some responsiveness to changes in the immediate environment of the adult.

[H1] Epigenetics in central control of reproduction

[H2] Genetics reveals epigenetically-regulated genes involved in pubertal timing

The timing of pubertal onset is clearly affected by the environment, but genetics also comprises a major determinant, with common and rare genetic variants having a role in the normal disparity seen in homogenous populations[149]. Unsurprisingly, many of these genetic variants are in key components of the HPG axis, such as genes encoding kisspeptin, GnRH, luteinizing hormone (LH) and follicle stimulating hormone (FSH) and their receptors[150]. Identification of genetic mutations associated with pubertal timing in the clinic has led to the discovery of novel epigenetically-regulated decisive factors, such as Makorin ring finger 3 (MKRN3) which represses the onset of puberty[151–154]. MKRN3 is an E3 ubiquitin ligase, and although its function in sexual maturation is not yet known, it is expressed highly in the arcuate nucleus (ARC) before puberty, drops markedly with pubertal onset and remains thereafter at low levels. Moreover this gene is imprinted and normally expressed only from the paternal allele due to methylation-mediated repression of the maternal allele[155]. Similar studies revealed mutations in Delta-like 1 homolog (DLK1) to be strongly associated with precocious puberty as well. *DLK1* is also a paternal-allele expressed imprinted gene whose expression levels in the hypothalamus fall at puberty[156]. The fact that both genes are programmed primarily via DNA methylation, and expressed from just one allele, renders them particularly susceptible to perturbation of epigenetic states with consequence on pubertal timing. Moreover DLK1, which is also known as preadipocyte factor 1, represses adipogenesis and is highly likely to be regulated by changes in the metabolic state, providing a novel mechanistic link between metabolism and reproduction[157].

GWAS have identified several factors associated with age at menarche, which might comprise novel epigenetically-regulated targets to mediate adaptive responses, especially given that many of them regulate body weight or metabolism[158–162]. One example *CRTC1* (which encodes CRTC1 or Transducer

Of Regulated CAMP Response Element-Binding Protein 1 (TORC1)) has been linked to age at menarche[162] and mediates the effects not only of nutritional status on metabolic programs, but also controls reproduction directly through activating *Kiss-1* and the genes that encode both subunits of FSH[163–166]. It is not yet clear to what degree CRTC1 activity is determined by epigenetic regulation, although it is apparently regulated by the deacetylase Sirtuin1[167].

[H2] Reproductive neuroendocrinology, epigenetics and connections with metabolism

The links between metabolic state, pubertal onset and reproductive function are well recognized[27,28,144,161,168]. Furthermore, changes in metabolite levels can directly alter the epigenome due to their role as cofactors for several key chromatin-modifying enzymes[169–171]; however, not much is known about how these two fields meet in the context of the neuroendocrine control of reproduction.

Pubertal onset hinges on a number of central neuroendocrine pathways that lead to reduced inhibition and increased activation of GnRH secretion, with pivotal stimulation by Kisspeptin[161,172–174]. Increased *Kiss1* promoter activity is mediated by a reduction in polycomb repressive complex (PRC) proteins CBX7 and EED, as well as GATAD1, which recruits KDM1A that removes activating H3 lysine 4 (H3K4) methylation[16,175]. Subsequently, two activating lysine methyl transferase complexes, KMT2A (MLL1), which catalyzes trimethylation of H3K4 at the *Kiss1* promoter, and KMT2C (MLL3) which binds a distal *Kiss1* enhancer, activate expression of the gene [17]. (**Fig 3**).

It is not yet known whether these neuroendocrine processes respond to external signals to mediate changes in the timing of puberty, other than for a mechanism that was suggested to involve Sirtuin enzymes, whose deacetylase activity is regulated by the metabolic state[18]. Prior to pubertal onset, the nutrient-sensitive histone deacetylase, Sirtuin1, represses *Kiss1* expression through interaction with the PRC complex[18]. Sirtuins require NAD+ as an essential cofactor, and in cases of nutritional excess, NAD+ is utilized heavily which might restrict Sirtuin activity and reduce this repression[169–171]. Therefore, in conditions of pre-pubertal over nutrition, the weakening of PRC-mediated inhibition that occurs towards puberty could be further facilitated by a drop in Sirtuin 1 activity, leading to precocious puberty. Conversely, under nutrition might have the opposite effect, delaying puberty due to enhanced Sirtuin 1-mediated repression of *Kiss1*[18].

Due to their utilization of α-ketoglutarate (αKG) as a cofactor, the DNA hydroxymethylase and/or demethylase TET enzymes are also sensitive to the metabolic state, and changes in αKG availability have been seen to regulate levels of DNA methylation and hydroxymethylation[176,177]. Increased GnRH expression during neuronal maturation in rhesus monkeys correlates with decreased DNA methylation at its promoter, suggesting relief of epigenetic mediated repression[19]. Furthermore, TET2 levels in the preoptic area increase through development in mice and this appears to facilitate GnRH expression. Although TET2 knockout did not affect pubertal onset, it resulted in reduced levels of circulating LH and reduced fecundity in males, suggesting a role in maintenance of GnRH neuronal function[20]. It is feasible that nutritional state might therefore also affect GnRH levels through moderating TET2 activity on the *Gnrh* promoter.

The pituitary gonadotropes respond to the metabolic state through direct sensing of altered glycaemic environment and resulting changes in glycolysis[24]. Ambient hyperglycaemic conditions lead to a major metabolic imbalance in these cells, including elevated levels of αKG and a drop in NAD+:NADH ratio, which affect TET and Sirtuin enzyme activity. Consequently, the gonadotrope epigenome undergoes a decrease in 5mC and increase in 5hmC DNA methylation, and elevated levels of histone acetylation[24]. The primary effect of these changes is a reduction in *Fshb* expression and circulating levels of FSH, which would be expected to affect female fertility[24]. In accordance with

these findings in mice, young girls with type 2 diabetes mellitus have steroid profiles that reflect low FSH levels, and worryingly, their menstrual dysfunction is reported not to improve following extensive anti-hyperglycemic treatment[178]. This outcome is highly suggestive of an epigenetic basis to their poor reproductive function, with implications for administering the most appropriate treatments.

[H2] Importance of the chromatin landscape

The functionality of an epigenetic modification can only be understood if studied in the relevant chromatin context. The mode of regulating a particular gene's expression is determined by the chromatin organization at its proximal promoter and often numerous distal transcriptional enhancers, which define how the gene can be activated or repressed. This chromatin landscape comprises the DNA and the packaging histone proteins, and how these are organized and modified (**Box 1**). The DNA sequence determines potential regulation by DNA methylation and hydroxymethylation, and also influences nucleosome positioning and the way in which the DNA is packaged into nucleosomes[179]. The nucleosome packaging varies also due to the inclusion of histone variants and covalent histone modifications, both of which can alter the DNA-histone interactions as well as the stability and mobility of the nucleosomes[25,180–182]. This organization is instrumental in determining the mechanisms that are involved in regulating gene transcription and expression levels and how they might be perturbed, and its study is thus essential to appreciate the possible function of any differential epigenetic modifications detected in large population screens[25,180,183,184].

The gonadotropin LH α and β subunit-encoding genes provide an example of how very different chromatin packaging explains not only their distinct expression levels and unique methods of regulation, but also their respective propensities to epigenetic regulation[15,25,185]. Transcription of *Cga*, which encodes the common gonadotropin α subunit, is initiated quite easily due to the activity of a cell-specific distal enhancer that directs open chromatin at the proximal promoter, leaving it free from nucleosomes in gonadotrope cells[25,26]. This chromatin organization allows unobstructed access of the regulatory transcription factors and general transcription machinery to the transcriptional start site (TSS) of the gene. The major barrier for up-regulating transcription is therefore traversing the first (+1) nucleosome, which is downstream of the TSS, and transcription is stimulated through targeting this nucleosome[25]. The +1 nucleosome is made less stable by incorporation of the histone variant H2A.Z, and this reduced stability helps RNAPII passage through the nucleosome, which increases rates of transcription[25]. The transcriptional up-regulation of this gene by GnRH also involves induction of several activating histone modifications, including H3 phosphorylation and acetylation, which reduce the histone tail-DNA interaction, likely also contributing to the transcription efficiency[14,25,183]. (**Fig 3**).

The potential regulation by DNA methylation of these genes is also distinct. In contrast to *Cga*, which virtually lacks CpG sites on its proximal promoter, *Lhb* is tightly regulated by DNA methylation and the TET enzymes[23]. In gonadotrope precursor cells, expression of *Lhb* is repressed by TET1 through non- catalytic mechanisms. However, TET1 expression is down regulated during development, and in fully-differentiated gonadotrope cells, its place on the *Lhb* gene promoter is replaced by TET2 which catalyzes the activating hydroxymethylation at this locus[23]. (**Fig 3**).

Also in marked contrast to *Cga*, the *Lhb* promoter is packaged in a nucleosome, which covers the binding sites for the crucial *Lhb* activating transcription factors, Sf-1, Pitx1 and Egr-1[15,25]. Expression of *Lhb* thus requires remodelling of the promoter chromatin to allow transcription initiation. This remodelling involves incorporation of H2A.Z into the promoter nucleosome, which increases its mobility on the DNA and facilitates transcription factor binding[15]. This nucleosome is also covalently modified following gonadotrope exposure to GnRH, which increases recruitment of menin, a key

component of the KMT2A and/or KMT2B methyl transferase complex that is responsible for the activating H3K4me3 modification at the gene promoter[21]. (**Fig 3**).

The chromatin landscape of *Fshb* has yet to be determined in such detail, but earlier studies reported that this gene is repressed in partially differentiated gonadotrope cell lines by class I and class II histone deacetylases (HDACs) whose actions are overcome by GnRH[22,186–188]. This repressive role for HDACs has been confirmed more recently also in primary gonadotrope cells from sexually immature mice, in which GnRH was seen to increase levels of histone acetylation at the *Fshb* gene promoter (L.P and P.M unpublished work). The repressive actions of HDACs on this gene and the ability of GnRH to overcome their actions likely comprise a key repressive mechanism that is lifted at the time of puberty when GnRH levels increase. (**Fig 3**). Further studies are required to understand the role of these and other epigenetic-driven mechanisms that mediate adaptive changes in reproductive function in response to the environment.

[H1] Identifying the epigenetic drivers

[H2] Reproduction, epigenetics and ageing: cause and effect

One of the shortcomings of trying to understand phenotypic variation by looking at human cohort methylome data, is being able to decipher cause from effect[189]. Methylome analyses have been performed in girls and boys across puberty using peripheral leukocytes or blood mononuclear cells, and in one study a causal relationship between the changes in DNA methylation and secondary sexual characteristics was inferred[190]. However, gonadal steroids are known to modify the epigenome[29–31], and two such studies reported particular enrichment for differential methylation at regions close to estrogen receptor binding sites or E2-responsive genes, which suggests that the altered methylation might be mediated by the increase in steroids[191,192]. As noted by these authors, it is not possible to determine from these observations whether the epigenetic changes are the result or the cause of the hormonal changes. Bessa and colleagues[192] also examined changes in CpG methylation in individuals with central precocious puberty (CPP), and identified 48 hypermethylated ZNF protein-encoding genes; which they acknowledged might contribute to CPP, but equally might result from functional changes in the genetic network underlying this condition.

Global levels of DNA methylation normally change over the lifespan, known as epigenetic drift or ageing[193,194], and this too is affected by activity of the reproductive axis. Some CpG sites show more variation over time than others, and when taken together they reflect quite precisely the ageing process. This observation has led to the development of predictive tools of chronological age, termed 'epigenetic clocks'[195,196]. The tick-rates of such epigenetic clocks are affected by various factors including environmental influences, and they correlate remarkably well with general health[195,197–200]. Importantly, the pace of epigenetic ageing is altered following puberty and menopause, and in certain tissues specifically in response to estradiol[32–34,201,202]. Thus, early-life environmental exposures or events that alter reproductive trajectories, including age at puberty or circulating hormone levels, would likely affect epigenetic ageing. So here too, differences observed in the methylome might reflect the altered reproductive function but are not necessarily responsible for the individual's phenotype. Deciphering how these changes in DNA methylation profiles relate to environmentally-modified reproductive strategies and the molecular mechanisms involved, requires additional experimental approaches, first to address whether the modification is in fact altered in the functional tissue and cell type that determines the reproductive phenotype, and then to demonstrate its role. (**Fig 4**).

[H2] Animal models, tissue accessibility and functional adaptive strategies

DNA methylation patterns vary between tissues, and in easily-attainable proxy tissues are not necessarily the same as in the relevant functional tissues[89,203,204]. Animal models can therefore be valuable for dissection of the relevant tissue, further facilitated by expression of fluorescent markers that allow isolation of specific cell populations. Enrichment of specific cell types is especially important for epigenetic profiling in heterogeneous tissues such as the pituitary[23,205], where the gonadotrope cells make up only a small percentage (3–10%) of the gland[139]. Without such isolation of the particular cell type, erroneous assumptions about differential epigenetic modifications can be made following changes in the relative population sizes rather than epigenetic changes *per se* in the cells of interest.

Animal models also present some of the complexities of the physiological and metabolic backgrounds that might mediate the adaptive response. Their energetic state can be manipulated, to some degree, to mimic specific human environments, although the parallels here are clearly limited, and new animal models that more accurately represent human biology are needed. Among rodents, the spiny mouse (*Acomys cahirinus*) is unique in experiencing human-like menstruation, and also post-natal maturation of the adrenal *zona reticularis*, mimicking changes in circulating adrenal androgen levels that occur during adrenarche[206,207]. Such characteristics suggest that this species might be more suitable for study of reproductive and adrenal function than commonly used rodent models. Mouse lemurs (*Microcebus spp*.) have also been heralded recently as a particularly suitable new model for studying human physiology and disease[208,209]; they share advantages of mice due to their small size, and breeding rates and capacities but, as primates, are genetically closer to humans.

Adopting new research models presents many technical drawbacks and hurdles, and ethical opposition, especially for primate research[209]. However, the information that appropriate animal models can contribute to identifying the mechanisms responsible for adaptive responses is invaluable, evident in our study on the Bangladeshi migrant populations in which we integrated findings from methylation analysis of human buccal DNA and a mouse model of early-life stress to mimic challenges of the Bangladeshi environment In this way we were able to identify altered epigenetic signatures of several genes that regulate ovarian follicle recruitment and appear to explain the depleted ovarian reserve in the women who spent their childhoods in Bangladesh (B.B.-S., L.P., K. B., G.L., R.D.E., R.S., G.R.B., P.M. unpublished work).

[H2] Determining a function for the epigenetic modification

Given the constant dialogue between the epigenome and reproductive axis, it is important that we verify whether a particular modification that is differentially-associated with a phenotype has a functional consequence. Discerning the importance of differential modifications at a specific genomic *locus* is usually challenging, unless found at known gene regulatory enhancers or promoters, which is highly indicative of function. Understanding the role of a modification on the expression of a particular gene requires knowledge of the chromatin landscape governing its transcription. High-precision mapping of the nucleosome positions, identification of the regulatory proteins that bind this genomic region and how this binding is altered by the modifications, as well as an understanding of the higher order chromatin structure and the role of long non-coding RNAs, all need to be considered (**Box 1**). An overview of these techniques is beyond the scope of this Review, but it should be emphasized that chromatin organization at gene regulatory regions can vary considerably between expressing and non-expressing cells, as at the *Cga* proximal promoter, which is devoid of a nucleosome only in gonadotropes[25]. This type of analysis must therefore be performed in an appropriate cell type that normally expresses the gene of study.

Once the chromatin landscape is known, its manipulation, for example using CRISPR-Cas9 based targeting approaches, can be used to establish functionality in cultured cells, and potentially also in animal models[210–212]. For greater precision, single-molecule approaches *in vitro* can reveal exactly how epigenetic modifications affect the interactions of packaging and regulatory proteins with a specific DNA sequence. After reconstituting nucleosomes on the regulatory DNA to recapitulate the native chromatin landscape, the DNA can be 'un-zipped' using an optical trap, to reveal both the force of interaction at pico-Newton scale, and the protein position on the DNA at nearly base -pair resolution[180,213]. (**Fig 4**). In this way, we have deciphered the function of H2A.Z at the gonadotropin genes[25] and revealed how a transcription factor binds chromatin-packaged DNA at the proximal promoter of *Lhb*[15,214]. This approach has the further potential to assess how epigenetic modifications affect RNAPII progression along the DNA, the effects of DNA and histone modifications as well as additional histone variants on nucleosome stability and mobility, and how these processes regulate the recruitment and effects of regulatory DNA-binding transcription factors. Clarification of the impact of epigenetic modifications that participate in adaptive reproductive strategies, at this level of resolution, is essential to complete our understanding of reproductive plasticity, and could facilitate the development of targeted approaches for modifying reproductive function.

[H1] Conclusions

The field of biological regulation is at one of its most exciting times. Human reproductive phenotypes are shaped by the environment and adult reproductive function can be reprogrammed following events during key developmental stages of early-life, as discussed here. Moreover, there is an expanding list of ways in which the chromatin can be modified through newly-discovered epigenetic modifications and regulatory non-coding RNAs that alter chromatin structure, providing seemingly endless mechanisms through which physiological function might be modified in response to external cues. Challenges ahead include integrating the roles of these potential regulators into our understanding of how the reproductive endocrine system operates normally and in adaptive responses to changes in the environment.

In a wider perspective, the environment in the developed world has changed dramatically since the industrial revolution, and is continuing to do so as technological advances alter fundamentally our life styles. Changes in quantity and quality of our nutrition, pathogen exposure, levels of physical activity and stress, surely affect our physiology directly, but might also provoke such adaptive responses in reproductive function, with important implications for human health. Beyond determining the start and end of the reproductive life-span and rates of fertility, consequences extend to the broad impact of hormonal exposure and epigenetic ageing on health and senescence more generally. Understanding these complex aspects of human health is thus crucial and will require extensive, multi-pronged approaches, as highlighted in this review. On the positive side, epigenetic modifications can potentially be targeted, so elucidating the mechanistic basis of these adaptive processes at a molecular level could open the way for developing tools to modify a variety of reproductive phenotypes.

Acknowledgements

This work was supported by grants from BBSRC/ESRC (grant ES/N000471/1 to G.R.B., R.S. and P.M.) and Israel Science Foundation (grants 1850/17 to P.M. and 1782/17 to A.K.).

Author contributions

B.B.-S., L.P., and S.R. researched data for the article and contributed to discussion of the content. P.M. wrote the article with considerable input from G.R. B., R.S. and A. K. who also researched the data and made substantial contributions to the discussion of content. All authors reviewed and/or edited the manuscript before submission.

Competing interests

The authors declare no competing interests.

Peer review information

Nature Reviews Endocrinology thanks the anonymous, reviewer(s) for their contribution to the peer review of this work.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Box 1: Determinants of the regulatory epigenetic landscape

The nucleotide sequence of DNA affects its mechanical properties, thus influencing nucleosome positioning, mobility and stability. The CpG content at regulatory DNA determines its potential regulation by DNA methylation and hydroxymethylation. DNA methylation recruits repressive protein complexes, so is usually inhibitory when occurring at gene promoters and enhancers, but likely has different roles in gene bodies. Hydroxylation of methylated cytosines can lead to active or passive demethylation, so facilitates transcription[179,215].

Nucleosome packaging of the DNA inhibits accessibility to regulatory proteins. For transcription to occur, nucleosomes must usually be destabilized, e.g. through incorporation of histone variants and covalent modification. This also facilitates nucleosome movement or eviction by ATP-dependent chromatin remodelers[182,216].

Histone variants alter various characteristics of the nucleosome: H2A.Z and H3.3 are histone variants commonly found at transcriptional start-sites of actively transcribed genes. H2A.Z incorporation increases nucleosome mobility and decreases stability. The role of H3.3 in transcription is less clear, but it was proposed to enhance accessibility of the chromatin to transcription factors[15,25,217,218].

Multiple histone modifications have diverse roles: Covalent histone modifications can signal recruitment of regulatory proteins, while some modifications alter the histone charge, possibly affecting interaction with the DNA or other histones. Histone acetylation is found in broad regions of active promoters and enhancers, and regulated by a number of histone acetyltransferases and deacetylases. Other histone modifications, like methylation, are more localized, catalyzed by fewer enzymes, and impart distinct effects depending on the number of modifications and specific histone residue targeted[219].

Functional enhancer RNAs: Transcriptional enhancers are often transcribed into noncoding RNA (eRNA), whose functions are beginning to emerge, but could vary in different contexts. Some eRNAs have been shown to regulate the proximal promoter chromatin landscape profoundly and play a role in enhancer-promoter DNA looping[26,183].

Higher-order chromatin: Chromatin 3D organization creates domains which both limit and mediate regulatory interactions. These domains change through development, and determine long-range transcriptional regulation[220].

Box 2: Glossary of Terms

5mC and 5hmC: 5-methylcytosine or 5-hydroxymethylcytosine modifications of the DNA.

Adrenarche: post-natal maturation of the adrenal gland which occurs almost uniquely in humans at ages 5-8 years and results in activation of 17,20-lyase activity and decreased 3β-hydroxysteroid dehydrogenase activity in the *zona reticularis,* greatly increasing circulating levels of DHEA and DHEAS.

Catch-up growth: a period of rapid growth that often occurs in children after their removal from growth-inhibiting conditions, and allow attainment of the expected final height.

Chromatin: packaging of the DNA around the histone proteins, the nature of which determines DNA accessibility.

CpG: a cytosine nucleotide followed by a guanine nucleotide, in the 5' to 3' direction.

CPP: central precocious puberty, when secondary sexual features appear before 8 years of age due to early activation of the HPG axis and elevated circulating gonadotropin levels.

DHEA and DHEAS: dehydroepiandrosterone and dehydroepiandrosterone sulfate: "weak" androgens (i.e. they bind the androgen receptor with low affinity) produced in humans largely by the adrenal glands following adrenarche.

DOHaD: developmental origins of health and disease.

FSH: follicle stimulating hormone, the pituitary hormone that stimulates follicle growth in females and conversion of weak androgens to strong androgens or estrogens in both sexes. It is composed of a common α-subunit (encoded by *CGA*) and a hormone-specific β-subunit (encoded by *FSHB*).

GnRH: gonadotropin releasing hormone, the hypothalamic hormone that stimulates the pituitary to produce and release the gonadotropins, LH and FSH.

Gonadotrope: the pituitary cell that synthesizes and secretes the gonadotropins, LH and FSH.

Gonadotropins: the pituitary hormones, LH and FSH.

HATs and HDACs: families of enzymes acting as histone acetyl transferases (HATs) or histone deacetylases (HDACs).

HPA axis: hypothalamic-pituitary-adrenal endocrine axis that regulates adrenal function, homeostasis and the stress response.

HPG axis: hypothalamic-pituitary-gonadal endocrine axis that regulates reproductive function.

KMT and KDM: families of enzymes acting as lysine methyl transferases (KMTs) or lysine demethylases (KDMs).

LH: luteinizing hormone, the pituitary hormone that stimulates ovulation and *corpus luteum* formation and progesterone synthesis in females, and androgen synthesis in both sexes. It is composed of a common α-subunit (encoded by *CGA*) and a hormone-specific β-subunit (encoded by *LHB*).

Menarche: the first cycle of menstrual bleeding in girls

NAD^+/NADH: Nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH); involved in electron transfer in cell metabolism, they also act as cofactors for many enzymes, including the Sirtuin histone deacetylases.

PRC: polycomb repressive complex which shuts down gene expression primarily through catalyzing repressive histone methylation.

Puberty: the process of sexual maturation during which the HPG axis is activated, the gonads start to produce sex-specific steroids in large amounts and the potential for sexual reproduction is attained.

RNAPII: RNA polymerase II that transcribes most protein-coding genes.

TET enzymes: Ten-eleven translocation enzymes that catalyze the conversion of 5mC to 5hmC, as well as other less common modifications (5-formylcytosine and 5-carboxylcytosine) which can lead to demethylation.

αKG: α-ketoglutarate (2-oxoglutarate) is produced during glucose metabolism following the deamination of glutamate, and is a crucial cofactor for several chromatin modifying enzymes, including the TET enzymes and most of the histone demethylases.

Figure Legends:

Figure 1: Life history theory of environmentally-induced reprogramming via the endocrine system. In response to changing environmental conditions and in the context of existing resources, resource allocation can be reprogrammed through the endocrine system to balance or shift between growth, homeostasis and reproduction, with the brain playing a central role, due to its ability to regulate all three endocrine axes.

Figure 2: Key early-life developmental stages in childhood and windows of susceptibility for reproductive axis re-programming. Fluctuations in pulsatile GnRH (top: adapted from[128]) and steroid levels (bottom: adapted from[221]) in boys and girls from late fetal development through sexual maturation; DHEA levels (adapted from[222]) are shown from birth (dashed line). These stages of hormonal fluctuations (shaded) reflect periods of changing epigenetic and gene expression networks, likely rendering them particularly vulnerable to modification. GnRH: gonadotropin releasing hormone; HPG: hypothalamic-pituitary-gonadal; T: testosterone; E2: estradiol; DHEA: dehydroepiandrosterone.

Figure 3: Sites of epigenetic regulation reported in the central control of reproduction. Expression of the hypothalamic *KISS1* and *GNRH* genes is inhibited in their respective neurons by factors shown in red. Activation of these genes involves removal of these repressors, and implementation of the activating epigenetic marks shown in green (most of the enzymes specifically responsible have yet to be identified). Similarly in the pituitary gonadotropes, a number of inhibitory factors (in red) have been identified that repress expression of the *CGA*, *LHB* and *FSHB* genes, and after their removal, activating marks (in green) are associated with gene expression. Data are taken from mouse model (refs in the main text).

Figure 4: Integrating experimental approaches to understand the role of the epigenome in adaptive reproductive strategies in humans. (1) Association of methylation data with reproductive phenotypic variation in human cohort studies can be combined with (2) animal models to verify modification in the functional tissue. (3) Cell models can then be used to characterize the chromatin at this genomic region and determine which genes are affected. (4) Subsequently, *in vitro* studies, preferably at the single-molecule level, can reveal the actual function of the epigenetic modification in terms of structural characteristics of the nucleosome, transcription factor binding and progression of the polymerase along the DNA. Understanding phenotypic variation in reproductive function and how this is determined by early-life experiences through changes in the epigenome will inform the

clinician and could lead to novel approaches for intervention of reproductive function and associated health benefits.

Population	Location	Subsistence Strategy	Environmental Stressors	Reproductive Characteristics*	Refs
Aymara	Bolivia	Agro-pastoralists	Seasonal nutritional stress and seasonally variable hard physical energy expenditure	Chronically lower levels of progesterone	[61]
Gainj	Papua New Guinea	Slash and burn horticulturalists	Nutritional, immunological, hard physical energy expenditure	Lower levels of reproductive steroid hormones, long menstrual cycles, lower rates of ovulation	[223]
Kaqchikel Maya	Guatemala	Subsistence agriculturalists	Nutritional, immunological, hard physical energy expenditure, psychological stress	Changes in luteal progesterone levels in relation to stress measured through cortisol levels.	[224]
Lese	Democratic Republic of Congo	Slash and burn horticulturalists	Nutritional, immunological, hard physical energy expenditure	Later puberty, chronically and seasonally lower levels of reproductive steroid hormones, fewer days menstrual bleeding, lower rates of ovulation	[59,225]
Migrant Bangladeshis	Sylhet, Bangladesh and London, UK	Developing country and industrialised nation	Immunological challenges during childhood	Shorter reproductive lifespan, chronically lower levels of progesterone and lower rates of ovulation in women who grow up in Bangladesh,	[5,6,56]
Polish	Poland	Rural farmers (non-mechanised)	Seasonally variable hard physical energy expenditure	Seasonally lower levels of progesterone	[226]
Tamang	Nepal	Agro-pastoralists	Seasonally variable hard physical energy expenditure	Chronically and seasonally lower levels of reproductive steroid hormones	[227]

Table 1: Examples of populations in energetically-demanding environments demonstrating plasticity in female reproductive characteristics

*Reproductive characteristics compared to normative clinical standards

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Author notes

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Fig 1

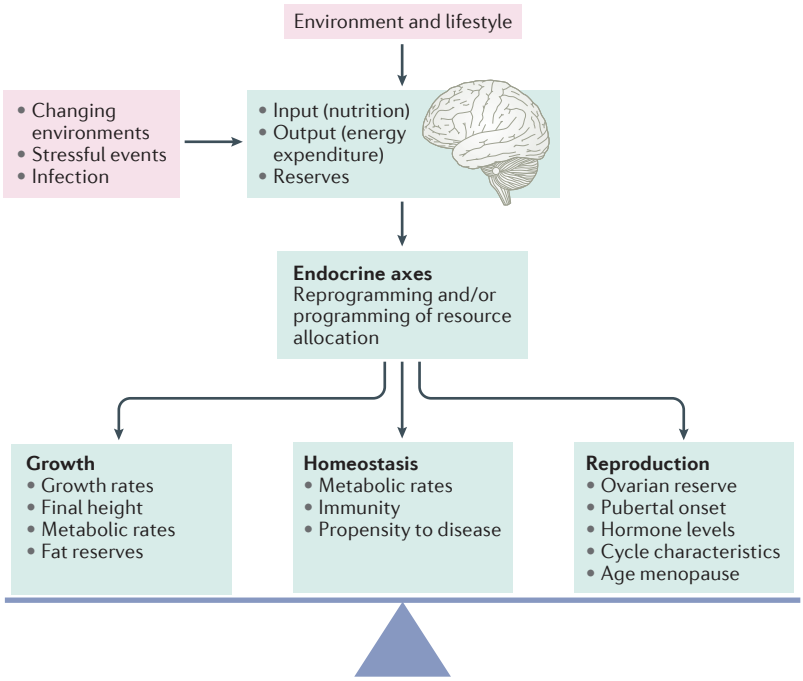


Fig 2

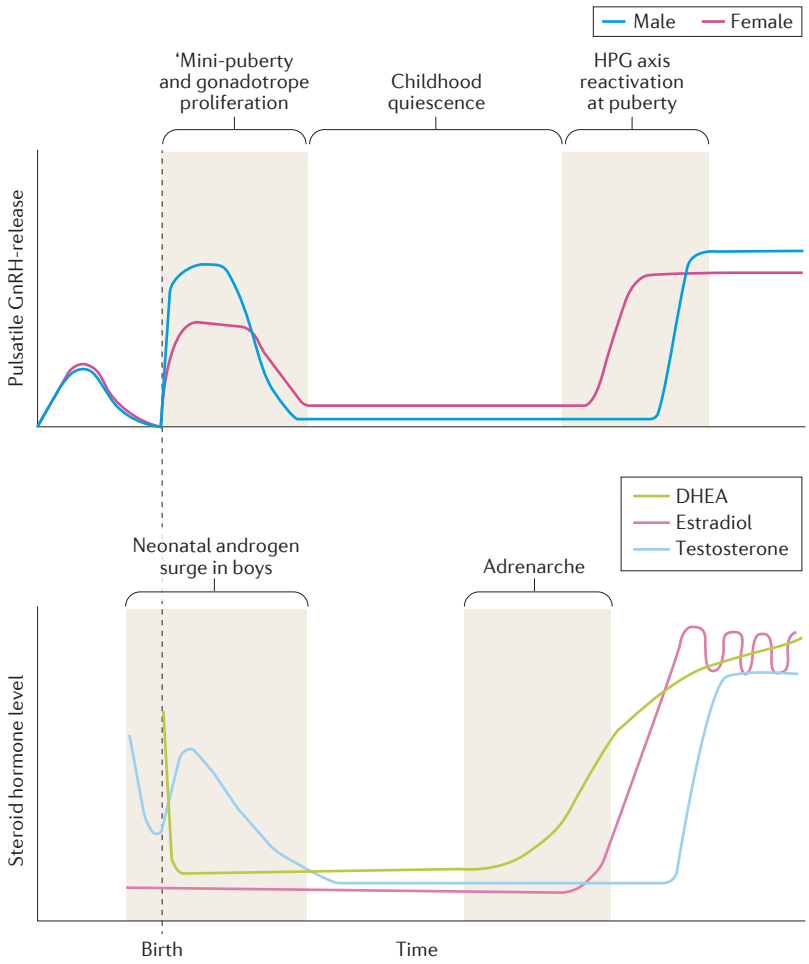
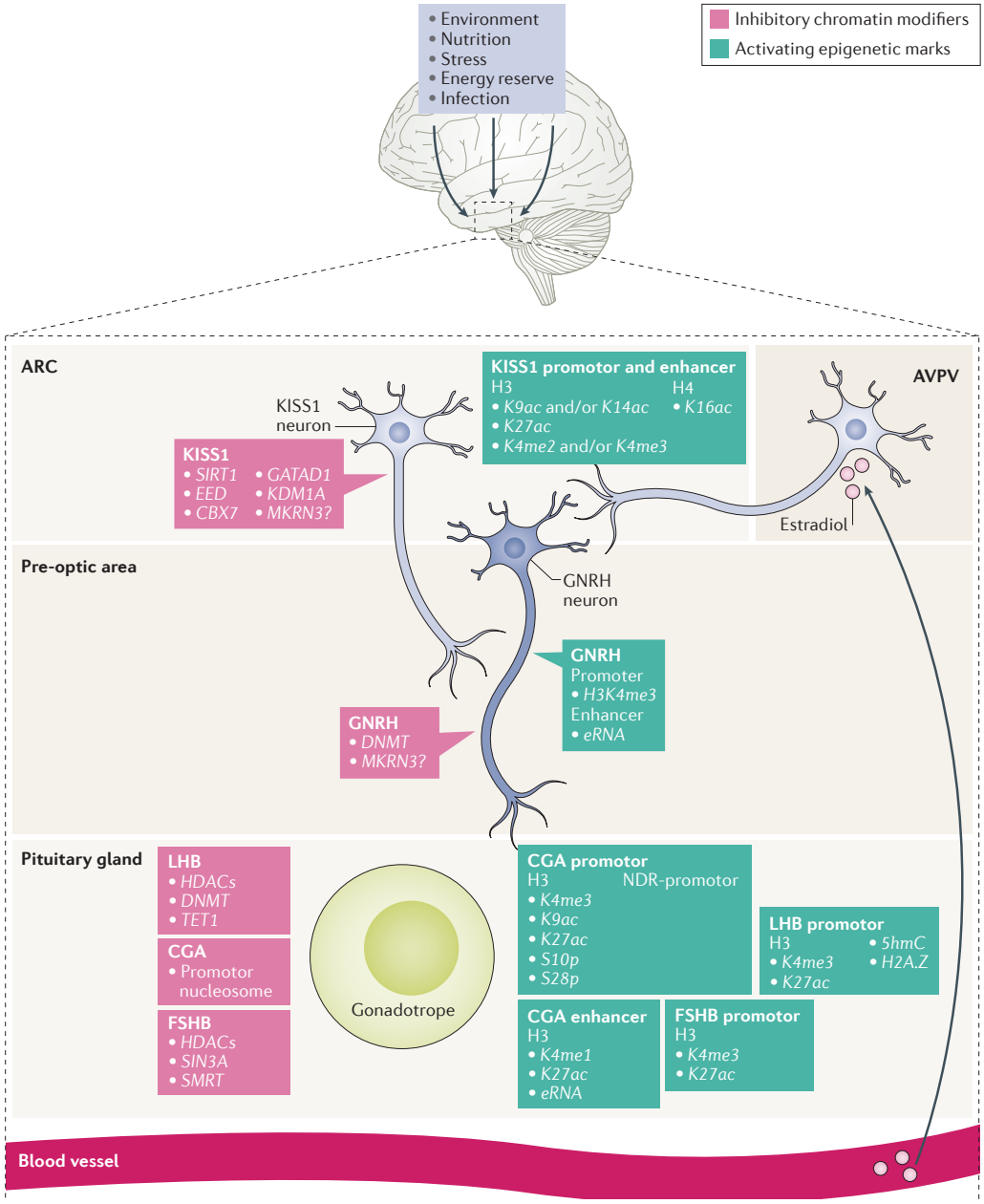

Male
Female
'Mini-puberty and gonadotrope proliferation
Childhood quiescence
HPG axis reactivation at puberty
Pulsatile GnRH-release
DHEA
Estradiol
Testosterone
Neonatal androgen surge in boys
Adrenarche
Steroid hormone level
Birth
Time

Fig 3


• Environment
• Nutrition
• Stress
• Energy reserve
• Infection
Inhibitory chromatin modifiers
Activating epigenetic marks
ARC
KISS1 neuron
KISS1
• SIRT1 • GATAD1
• EED • KDM1A
• CBX7 • MKRN3?
KISS1 promotor and enhancer
H3
• K9ac and/or K14ac
• K27ac
• K4me2 and/or K4me3
H4
• K16ac
AVPV
Estradiol
Pre-optic area
GNRH neuron
GNRH
Promoter
• H3K4me3
Enhancer
• eRNA
GNRH
• DNMT
• MKRN3?
Pituitary gland
LHB
• HDACs
• DNMT
• TET1
CGA
• Promotor nucleosome
FSHB
• HDACs
• SIN3A
• SMRT
Gonadotrope
CGA promotor
H3
• K4me3
• K9ac
• K27ac
• S10p
• S28p
NDR-promotor
LHB promotor
H3
• K4me3
• K27ac
• 5hmC
• H2A.Z
CGA enhancer
H3
• K4me1
• K27ac
• eRNA
FSHB promotor
H3
• K4me3
• K27ac
Blood vessel

NREND-19-080 Melamed 24|04|20

Fig 4