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Emerging concepts on the epigenetic and transcriptional regulation of the *Kiss1* gene

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ABSTRACT

Kisspeptin and its receptor have been implicated as critical regulators of reproductive physiology, with humans and mice without functioning kisspeptin systems displaying severe pubertal and reproductive defects. Alterations in the expression of *Kiss1* (the gene encoding kisspeptin) over development, along with differences in *Kiss1* expression between the sexes in adulthood, may be critical for the maturation and functioning of the neuroendocrine reproductive system and could possibly contribute to pubertal progression, sex differences in luteinizing hormone secretion, and other facets of reproductive physiology. It is therefore essential to understand how *Kiss1* gene expression develops and what possible regulatory mechanisms govern the modulation of its expression. A number of recent studies, primarily in rodent or cell line models, have focused on the contributions of epigenetic mechanisms to the regulation of *Kiss1* gene expression; thus far, mechanisms such as DNA methylation, histone acetylation, and histone methylation have been investigated. This review discusses the most recent findings on the epigenetic control of *Kiss1* expression in adulthood, the evidence for epigenetic factors affecting *Kiss1* expression during puberty and development, and findings regarding the contribution of epigenetics to the sexually dimorphic expression of *Kiss1* in the hypothalamus.

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1. Introduction

The brain governs puberty onset and fertility via its secretion of gonadotropin releasing hormone (GnRH), the master hormonal regulator of the neuroendocrine reproductive axis. Recently, it was discovered that one of the key upstream neural circuits involved in the control of GnRH secretion consists of hypothalamic neurons expressing the *Kiss1* gene and its protein product, the neuropeptide kisspeptin. Kisspeptin signaling has been implicated as an essential regulator of fertility and puberty in all mammalian species, including humans (de Roux et al., 2003; Kauffman, 2010; Pineda et al., 2010; Pinilla et al., 2012; Seminara et al., 2003). Alterations in the expression of *Kiss1* or kisspeptin over development, along with differences in expression between the sexes, during both key developmental periods and adulthood, may be a critical factor in the maturation and functioning of the neuroendocrine reproductive axis. Indeed, changes in the *Kiss1* system may contribute to the aspects of puberty onset or progression, sex differences in estrogen-induced luteinizing hormone (LH) secretion in adulthood,

and other facets of reproductive physiology. It is therefore essential to understand how and when *Kiss1* expression develops, and what possible regulatory mechanisms govern the development and regulation of the kisspeptin system (Semaan and Kauffman, 2010).

A number of recent studies have focused on the epigenetic regulation of *Kiss1* gene expression (Gill et al., 2012; Semaan et al., 2012; Tomikawa et al., 2012; Lomniczi et al., 2013). Epigenetics is a term that literally means acting 'above the genome' and refers to changes in gene activity and expression that cannot be attributed to DNA sequence alone, such as functional modifications to a chromosome that do not involve a change in the nucleotide sequence. Generally, the term epigenetics is used to describe alterations in gene expression potential that arise during development, differentiation, and/or under the control of environmental factors. Importantly, the control of gene expression via epigenetic mechanisms enables the integration of both intrinsically programmed and environmental signals, thus allowing for the ability to adapt to a changing environment by altering the activity of genes (Bernstein et al., 2007; Kooistra and Helin, 2012; Rando, 2012; Reik, 2007). This review begins with a brief summary of key concepts in kisspeptin/*Kiss1* biology and then discusses the current knowledge of the epigenetic control of *Kiss1* expression in response to adult sex steroid signals, during development, and with regards to the sexually dimorphic expression of *Kiss1* in the hypothalamus.

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2. Kiss1 gene expression in adulthood and development

2.1. Regulation of Kiss1 expression by sex steroids in adulthood

Recent studies have identified the importance of the *Kiss1* system for reproductive function in a number of mammalian species. In humans and mice, deletions or mutations in the *Kiss1* or kisspeptin receptor genes result in infertility and impaired pubertal maturation (Clarkson et al., 2008; d'Anglemont de Tassigny et al., 2007; de Roux et al., 2003; Seminara et al., 2003). Recently, however, there has been some controversy about the absolute need for kisspeptin for the initiation of puberty and there may be other neural factors acting upstream or in parallel that ultimately govern puberty onset (Guerrero et al., 2012; Mayer and Boehm, 2011). Regardless, it is well-established that exogenous treatment of peripubertal and adult rodents, ungulates, and primates with kisspeptin potentially increases circulating LH and follicle stimulating hormone (FSH) levels (Dhillon et al., 2007; Gottsch et al., 2004; Messenger et al., 2005; Navarro et al., 2005a,b; Shahab et al., 2005) and that the stimulation of gonadotropin secretion by kisspeptin is via direct activation of GnRH neurons (reviewed in (Hrabovszky et al., 2010; Kauffman, 2010; Pinilla et al., 2012)).

In rodents, *Kiss1*-expressing (and kisspeptin-immunoreactive) cell bodies are located in just a few discrete brain regions, including a small population in the medial amygdala (Kim et al., 2011) and two larger hypothalamic populations in the anteroventral periventricular nucleus and neighboring periventricular nucleus (AVPV/PeN) and the arcuate nucleus (ARC) (Gottsch et al., 2004; Kauffman et al., 2007a; Smith et al., 2005a). In non-rodent species, such as sheep and non-human primates, *Kiss1* gene expression and kisspeptin immunoreactivity have, for the most part, a similar distribution as in rodents, with expression localized to the pre-optic area (POA) and the ARC/infundibular nucleus (Kim et al., 2009; Ramaswamy et al., 2008; Shahab et al., 2005; Smith et al., 2007, 2010). Similarly, in humans, *KISS1* distribution is mainly in the infundibular nucleus (homologue of the ARC), and also found sparsely in the medial preoptic area (Romero et al., 2007).

In adult rodents, and other species, the level of *Kiss1* expression in the AVPV/PeN and the ARC is differentially regulated in response to gonadal sex steroids. Expression of *Kiss1* in the ARC is inhibited by sex steroids, while in the AVPV/PeN, *Kiss1* expression is stimulated by sex steroids (Smith et al., 2005a). The fact that the ARC *Kiss1* population is inhibited by sex steroids supports the hypothesis that this population has a role in mediating negative feedback effects of gonadal sex steroids on basal pulsatile GnRH secretion in both sexes. On the other hand, the stimulation of AVPV/PeN *Kiss1* neurons by sex steroids likely reflects this population's involvement in mediating estradiol's (E_2) positive feedback induction of the preovulatory LH surge, a sexually dimorphic event which only occurs in females.

2.2. Changes in Kiss1 expression during development and pubertal maturation

In adult animals, *Kiss1* is highly expressed in the hypothalamic AVPV/PeN and ARC regions, but this is not always the case earlier in development. In rodents, *Kiss1* expression is present in the brain embryonically and at birth, but only in the ARC region (Desrozières et al., 2012; Poling and Kauffman, 2012a), with no detectable AVPV/PeN *Kiss1* expression at this time (Semaan et al., 2010). In fact, neither *Kiss1* mRNA nor kisspeptin protein has been detected in the AVPV/PeN of mice or rats on the day of birth or several days thereafter. The earliest documented AVPV/PeN *Kiss1* expression in mice was recently shown, using *in situ* hybridization, to be around postnatal day (PND) 10 (Semaan et al., 2010), though this *Kiss1* expression was of very low magnitude compared to PND 12 and

PND 14, when moderately higher levels were observed. Another developmental study of kisspeptin protein staining in female mice found no kisspeptin-immunoreactive (ir) cells in the AVPV/PeN at PND 10, and only a small number of kisspeptin cells at PND 15, which was the next chronological age examined (Clarkson et al., 2009). Looking beyond the first two weeks of life, kisspeptin-ir cell number in the mouse AVPV/PeN was found to steadily and markedly increase from PND 15 to adulthood (assessed every 5 days of age) (Clarkson et al., 2009). Extending this protein data, we recently performed a detailed, day-by-day analysis of *Kiss1* mRNA expression in peripubertal female mice and found that *Kiss1* cell number in the AVPV/PeN dramatically increases from PND 15 through PND 28, at which point it resembles adulthood levels (Semaan and Kauffman, 2012).

Unlike in the AVPV/PeN, *Kiss1* is expressed in the rodent ARC during embryonic development, with detectable expression beginning around embryonic day 14 (Desrozières et al., 2012). Likewise, ARC *Kiss1* expression is readily detectable on the day of birth in mice and rats and persists throughout the remainder of postnatal and pubertal development (Cao and Patisaul, 2011; Cao et al., 2012; Poling and Kauffman, 2012b). However, the peripubertal profile of *Kiss1* gene expression in the ARC is not as clear cut as in the AVPV/PeN, and conflicting data exist regarding changes in ARC *Kiss1* expression during prepubertal and pubertal development. Some studies report no major differences or slight reductions in ARC *Kiss1* expression levels between juvenile and adult animals (Gill et al., 2010; Han et al., 2005; Navarro et al., 2012; Semaan and Kauffman, 2012), while other studies report small increases in ARC *Kiss1* expression around the time of early puberty (Bentsen et al., 2010; Takase et al., 2009). Thus, it is currently unresolved whether *Kiss1* expression in the ARC significantly changes during puberty.

2.3. Sex differences in Kiss1 expression

Kiss1-expressing neurons in the rodent AVPV/PeN are sexually differentiated, with pubertal and adult females possessing more *Kiss1* mRNA and more detectable *Kiss1* cells in this region than males (Kauffman et al., 2007a; Semaan et al., 2010). Similar sex differences have been reported for kisspeptin protein levels in the rodent AVPV/PeN (Clarkson and Herbison, 2006; Homma et al., 2009). In mice, *Kiss1* mRNA is first expressed in the AVPV/PeN around PND 10 in both sexes, but intriguingly, there is no sex difference in AVPV/PeN *Kiss1* expression at this age (Semaan et al., 2010). The *Kiss1* sex difference, however, is evident on PND 12, and becomes even more robust on PND 14 and 16 (Semaan et al., 2010). Thus, AVPV/PeN *Kiss1* expression first arises at the same age in both sexes (~PND 10), but the sex difference in *Kiss1* levels takes several more days to emerge, at least as assessed via *in situ* hybridization, suggesting that this sex difference may be influenced by regulatory factors affecting *Kiss1* gene expression.

Although activational effects of sex steroids transiently modulate *Kiss1* expression levels in adulthood, with circulating testosterone (T) or E_2 stimulating *Kiss1* expression in the AVPV/PeN (Smith et al., 2005b), the adult sex steroid milieu does not cause the observed sex differences in AVPV/PeN *Kiss1* expression (Kauffman et al., 2007a). This is evidenced by the fact that adult male and female rodents gonadectomized and treated with identical E_2 levels still display sexually dimorphic *Kiss1* expression in the AVPV/PeN (Adachi et al., 2007; Kauffman et al., 2007b; Semaan et al., 2010). Rather, the AVPV/PeN *Kiss1* sex difference, like many other sex differences, is permanently organized by sex steroid signaling early in postnatal development, around the time of birth. Detailed discussion of this “organizational” model of sexual differentiation of brain circuits has recently been reviewed elsewhere (Kauffman, 2010; Lenz et al., 2012; Semaan and Kauffman, 2010). In brief, the central tenet of this model posits that, during the postnatal “critical period”,

which in rodents is characterized as approximately the first week of postnatal life, males normally secrete elevated gonadal T, whereas females secrete less sex steroids at this time (Simerly, 1998). The presence of elevated levels of sex steroids in postnatal males causes many sexually dimorphic traits to develop to be male-like in adulthood, whereas the absence of sufficient levels of sex steroids in postnatal females causes their brains to permanently develop the female phenotype (Simerly, 1998, 2002).

Several studies have determined that the AVPV/PeN *Kiss1* system is organized postnatally by sex steroids. For example, castrating male rats at birth, to remove elevated sex steroid levels, causes a permanent feminization of the developing AVPV/PeN *Kiss1* system (Homma et al., 2009). Conversely, neonatal female rats treated once with T or E₂ during the critical period, to mimic a newborn male's steroid levels, exhibit a permanent reduction of *Kiss1*- or kisspeptin-expressing cells in the AVPV/PeN later in adulthood, similar to what is exhibited in normal adult males (Bateman and Patisaul, 2008; Homma et al., 2009; Kauffman et al., 2007b). Because postnatal E₂ treatment can, like T, permanently alter the development of the AVPV/PeN *Kiss1* system, it suggests that the postnatal masculinization of this system is likely mediated via neural aromatization of T to E₂. Importantly, the reduction of AVPV/PeN *Kiss1* expression in female rats that were neonatally treated with E₂ correlates with their inability to generate an E₂-mediated LH surge as adults (Homma et al., 2009), linking the sexually dimorphic *Kiss1* system with the sexually dimorphic LH surge event.

3. Epigenetic control of gene expression

What causes the changes in *Kiss1* gene expression during sexual differentiation, development, puberty, and adulthood? In some cases, the answer may lie in the global and temporal regulation of the structure of the DNA and protein complexes containing the *Kiss1* gene sequence, i.e., the *Kiss1* chromatin structure. Every cell within a multicellular organism possesses the same DNA sequence and, thereby, the entire genetic blueprint. However, the functional roles of different cells vary from tissue to tissue and even within tissue. These functional roles are controlled, by and large, through regulated differential gene expression. Differences in gene expression are mainly regulated by epigenetic mechanisms, where the DNA sequence itself is not modified, but factors acting “above” the genome – epigenetic factors – allow for changes in the level of gene expression (Bernstein et al., 2007; Goldberg et al., 2007; Jaenisch and Bird, 2003). The two most well-studied of these epigenetic mechanisms are histone modifications and DNA methylation, which alter gene expression by ultimately affecting chromatin structure and/or the ability of transcription factors to bind the DNA. There is also mounting evidence that these different epigenetic mechanisms themselves can functionally interact, which indicates that different epigenetic marks can function together to activate or repress gene expression (Goldberg et al., 2007; Rando, 2012).

3.1. Histone modifications

The term chromatin refers to DNA associating with proteins, called histones, to form a complex. It is largely accepted that an “open” or de-compacted chromatin conformation allows transcriptional machinery to access a gene and increase transcription, resulting in an increase in gene expression, while a “closed” or compacted chromatin conformation would have the opposite effect. A number of modifications to histones have been implicated in affecting the conformation of chromatin, and these include, but are not limited to, acetylation/deacetylation and methylation/demethylation of lysine residues comprising histone proteins

(Bernstein et al., 2007; Kooistra and Helin, 2012; Reik, 2007). Importantly, key changes in gene expression during development are often associated with these histone modifications, especially as cells differentiate from a stem cell (Reik, 2007).

Modifications to core histone proteins can result in either a compaction or de-compaction of chromatin, depending on both the specific residues where the modification occurs and the type of modification. Enzymes called histone methyltransferases mediate the process of histone methylation, usually modifying a single lysine on a single histone, with the effect of either activation or repression of transcription (Kouzarides, 2007). For example, methylation of histone H3 at lysine 27 (H3K27) or lysine 9 (H3K9) causes a silencing of gene expression, whereas histone methylation at lysine 4 (H3K4) activates gene expression (Reik, 2007). The H3K27 epigenetic mark is brought about through the polycomb group repressive complexes (PcG), which are comprised of protein complexes with methylase activity (Morey and Helin, 2010). The histone methylation actions of PcGs can cause genes to be held inactive prior to and/or during differentiation, until they are required at a later time period. In many cases, removal of silencing methylation marks, thought to be mediated by histone demethylases, is required for de-repression of gene expression. Indeed, the discovery of the first histone demethylase (LSD1) has paved the way for abundant research into histone methylation/demethylation mechanisms, and a number of studies are focusing on what influences histone demethylase activity and specificity (Kooistra and Helin, 2012; Kouzarides, 2007).

The other commonly studied histone modification, acetylation, is often associated with transcriptional activation (Kouzarides, 2007; Li et al., 2007). Unlike histone methylation, histone acetylation typically occurs at multiple lysines and is carried out by a number of histone acetyltransferases (HATs) (Li et al., 2007). It is thought that specific patterns of histone acetylation at multiple lysine residues can control the regulation of co-expressed genes and/or the cumulative effect of a number of modified lysines can control the function of this modification (i.e., the function of a single histone acetylation mark cannot be interpreted without understanding the combinatorial occurrences with acetylation marks at other lysine residues) (Li et al., 2007; Rando, 2012). Often, de-acetylation of histones results in transcriptional repression (Kouzarides, 2007; Li et al., 2007). Currently, there are three known classes of histone deacetylases (HDACs), which are involved in numerous signaling pathways and associated with repressive chromatin complexes (Kouzarides, 2007).

3.2. DNA methylation

The other well-studied epigenetic modification is DNA methylation at cytosine guanine dinucleotide (CpG) sites. DNA methylation has been implicated in a number of cellular functions and disease states, as well as the control of tissue specific gene expression, X-inactivation, and chromatin structure (Jaenisch and Bird, 2003). CpG methylation occurs throughout the genome, but frequently, the ability to regulate gene expression depends on the methylation status in gene promoter regions and other important regulatory regions, such as enhancers (Goldberg et al., 2007; Jaenisch and Bird, 2003). Methylated cytosines can either promote or block the recruitment of transcription factors to gene regulatory regions, often with the final effect of repressing gene transcription (Bernstein et al., 2007; Cedar and Bergman, 2012; Jaenisch and Bird, 2003). One way in which methylated DNA is recognized by cellular transcriptional machinery is via methyl-CpG binding proteins, which are thought to interact with HDACs (Bernstein et al., 2007; Cedar and Bergman, 2012; Jaenisch and Bird, 2003; Jones et al., 1998). Methylated cytosines can block DNA binding proteins, such as transcriptional activators or repressors, and thus, affect gene

transcription in this manner (Eden et al., 2001; Jaenisch and Bird, 2003; Jones and Takai, 2001).

DNA methyltransferases (DNMTs) establish DNA methylation patterns during development and under certain conditions, such as stress, in response to environmental signals, or to hormones and other signaling molecules. DNMTs (such as DNMT1) can have either have a DNA methylation maintenance function, insuring the previous methylation mark is maintained through cell division, or (as with DNMT3A) a de novo function, responsible for the addition of new methyl groups to cytosines (Jaenisch and Bird, 2003). Importantly, DNMTs are thought to be able to interact directly or indirectly with histone modifying enzymes, and thus, are able to sense other types of epigenetic marks and concomitantly control gene expression (Cedar and Bergman, 2012). DNA methylation is dynamic across development, and methylation marks can also be removed, although the mechanisms by which this occurs is still under debate. It has been suggested, for some time now, that DNA demethylation may proceed via DNA repair mechanisms (Reik, 2007; Wu and Zhang, 2010) and recently, a 5-methylcytosine hydroxylase, TET1, has been shown to promote DNA demethylation in mammalian cells through a process that requires the base excision repair pathway (Guo et al., 2011).

4. Epigenetic involvement in the sex steroid regulation of *Kiss1* expression in adulthood

As mentioned above, in pubertal and adult animals, *Kiss1* gene expression in the AVPV/PeN is robustly upregulated by E_2 and T, while in the ARC, the opposite is true: sex steroids inhibit ARC *Kiss1* expression (Smith et al., 2005b; Smith, 2008). Because epigenetic modifications to histones are tightly linked to gene expression, it is possible that the regional difference in *Kiss1* response to E_2 in the AVPV/PeN and ARC is due to different epigenetic modifications occurring in each region. For example, one might predict that, in the AVPV/PeN, E_2 induces an opening of the chromatin, perhaps by increasing histone acetylation, thereby promoting increased *Kiss1* transcription, while in the ARC, E_2 causes a closing of chromatin, perhaps via histone deacetylation, resulting in decreased *Kiss1* gene expression. This possibility was tested in a recent study focusing on the potential involvement of histone H3 acetylation in the differential regulation of *Kiss1* gene expression in the AVPV/PeN and ARC. Utilizing chromatin immunoprecipitation (ChIP) on micropunched brain tissue, Tomikawa and colleagues found that histone H3 acetylation of the *Kiss1* promoter region increased in the AVPV/PeN in response to E_2 , coinciding with increased *Kiss1* expression in this brain region. Conversely, a reduction of histone H3 acetylation of the *Kiss1* promoter was observed in the ARC in response to E_2 , coinciding with decreased *Kiss1* expression in this brain area (Tomikawa et al., 2012) (Fig. 1A). Not only did E_2 treatment modify histone H3 acetylation at the *Kiss1* promoter, but in the ARC, E_2 -induced changes in histone H3 acetylation were also found in a 5' region upstream of the *Kiss1* promoter (increased acetylation), as well as at a 3' intergenic region (decreased acetylation) located downstream of the last exon of the *Kiss1* gene. This interesting finding suggests that these non-promoter gene regions may be important for the regulation of *Kiss1* expression. Furthermore, this study confirmed, utilizing ChIP assays, that E_2 treatment increased the association of estrogen receptor α (ER α) in the *Kiss1* promoter region in AVPV/PeN tissue, but not in the ARC (Tomikawa et al., 2012). These data coincide well with the previous finding that *Kiss1* in the AVPV/PeN is regulated by ER α via a "classical" signaling mechanism, while a non-classical ER α signaling mechanism of regulation of *Kiss1* expression occurs in the ARC (Gottsch et al., 2009).

Tomikawa and colleagues expanded their investigation on the upstream 5' region and 3' intergenic area of the *Kiss1*

gene, examining these regions as possible enhancer regions that might increase *Kiss1* transcription via chromatin loop formation. Enhancer regions are generally located far away from the promoter of genes, but can affect transcription via physical bending or loop formation with promoters via the association of proteins involved in transcription. Utilizing a chromatin conformation capture assay, the investigators found that, in the AVPV/PeN, E_2 treatment enhances the association of the *Kiss1* promoter with the 3' intergenic region, as well as increases the association of the 5' region with the promoter, thereby forming a chromatin loop. In the ARC, these two DNA regions interacted with the *Kiss1* promoter regardless of E_2 treatment (Tomikawa et al., 2012). Thus, in the ARC, these 2 non-promoter regions may not be critical for E_2 's downregulation of *Kiss1* expression.

To further study the role of the 3' downstream 10 kb region of the *Kiss1* gene, Tomikawa et al. developed novel transgenic mouse models lacking this specific gene region. These transgenic mice either contained both the 22 kb upstream and 10 kb downstream regions, or only the 22 kb upstream region (lacking the 10 kb 3' downstream sequence). Additionally, both transgenic mice contained an AcGFP sequence in the second exon of the *Kiss1* gene, to act as a reporter of *Kiss1* expression. In transgenic mice lacking the 3' 10 kb region, GFP levels were low in the AVPV/PeN, even after E_2 treatment (which normally increases *Kiss1* expression in this area), compared to wildtype mice with an intact 3' 10 kb downstream region. Thus, this 3' downstream region appears to be critical for ability of E_2 to increase *Kiss1* expression in the AVPV/PeN and is likely functioning as an enhancer (Fig. 1B). However, more studies are needed in order to pinpoint the exact location of the enhancer in this rather large region, what other proteins are involved, and where exactly this enhancer interacts at the promoter (presumably at or near the estrogen response elements) (Fig. 1B).

It is worth noting that, in this particular study, the contributions of DNA methylation to the epigenetic control of differential *Kiss1* expression in the AVPV/PeN and ARC were not studied in detail, as these investigators noted only small differences between the ARC and AVPV/PeN in CpG methylation in the first six CpGs examined upstream of the first exon (Tomikawa et al., 2012). However, because no other *Kiss1* CpG sites were examined, the contributions of DNA methylation to *Kiss1* expression in adulthood cannot be ruled out and will need to be addressed in future studies. Regardless, this timely study demonstrated that E_2 -induced changes in histone H3 acetylation in the AVPV/PeN and ARC may contribute to differential *Kiss1* expression in these brain regions in response to E_2 . Additionally, a newly-identified DNA region downstream of the *Kiss1* gene coding region appears to act as an enhancer, especially for the E_2 -induced *Kiss1* expression increase in the AVPV/PeN. In the future, it will be important to explore how E_2 is interacting with chromatin to cause this looping effect and what other proteins are involved. Upcoming investigations may also examine, in greater detail, the 5' upstream region's involvement in the regulation of *Kiss1* expression in the AVPV/PeN and ARC.

5. Epigenetic mechanisms involved in *Kiss1* pubertal maturation

Kisspeptin has been implicated as a fundamental stimulator of GnRH secretion, thus possibly playing a role in the enhanced GnRH secretion that occurs during pubertal progression. Yet, the many endogenous regulatory mechanisms governing changes in the kisspeptin system during the pubertal period remain unknown. As mentioned earlier, it is well-known that AVPV/PeN *Kiss1* expression increases markedly from the juvenile stage to adulthood, whereas conflicting reports exist about whether or not *Kiss1* expression in

Fig. 1. (A) A schematic depicting how histone acetylation levels change with estradiol (E_2) exposure in the AVPV/PeN and ARC of mice (Tomikawa et al., 2012). E_2 causes an increase in *Kiss1* expression in the AVPV/PeN while simultaneously causing a decrease in *Kiss1* expression in the ARC. Higher levels of acetylated histone H3 are associated with the *Kiss1* gene in the AVPV/PeN after E_2 treatment, while in the ARC, lower levels of histone acetylation in the presence of E_2 lead to compacted chromatin and reduced *Kiss1* gene expression. (B) Cartoon model depicting chromatin loop formation in the AVPV/PeN after E_2 treatment. A portion of the DNA region located 3' of the last exon of the *Kiss1* gene (extending over 10 kb downstream) appears to act as an enhancer for *Kiss1* expression in the AVPV/PeN. When no E_2 is present, the enhancer loop does not form, but in the presence of E_2 , the 3' region interacts in the putative promoter region of the *Kiss1* gene, presumably via the interaction of transcription factors, such as estrogen receptor α .

the ARC increases significantly during puberty. Because kisspeptin signaling is fundamental for reproduction, it is likely that epigenetic modulation of *Kiss1* expression may occur during puberty onset and/or the subsequent pubertal transition.

A recent study in rats from Sergio Ojeda's group has implicated the involvement of genes comprising the repressive polycomb group (PcG) protein complexes in the inhibition of pubertal onset, via their negative regulation of the *Kiss1* gene (Fig. 2A) (Lomniczi et al., 2013). The authors identified, via microarray, a pubertal

decrease in two genes (*Eed* and *Cbx7*) that are important for the repressive function of PcGs on expression of other target genes. This pubertal decrease in *Eed* and *Cbx7* expression coincided with an increase in methylation of their respective promoters (Lomniczi et al., 2013). When a DNA methyltransferase inhibitor was administered to rats 6 days prior to normal pubertal onset, DNA methylation of the *Eed* and *Cbx7* genes was blocked and their expression remained elevated, correlating with a delay in pubertal onset (Lomniczi et al., 2013).

Fig. 2. (A) A schematic illustrating that genes involved in PcG protein complex function are repressed at pubertal onset to allow transcription of the *Kiss1* gene. During the pre-pubertal period, *Kiss1* expression is inhibited but the *Eed* and *Cbx7* (components of PcG complexes) genes are turned on, allowing PcG complexes to suppress *Kiss1* expression. Around the time of pubertal onset, the *Eed* and *Cbx7* promoters become methylated, causing a decrease in their expression. This causes the PcG complexes to have lower association with the *Kiss1* gene, allowing the *Kiss1* gene to be turned on by epigenetic modifications, and thereby initiate puberty (Lomniczi et al., 2013). (B) Diagram depicting the possibility that *Kiss1* expression during puberty may be regulated by demethylation of histone H3 at lysine 9 (H3K9) (Gill et al., 2012). Preliminary data suggest that H3K9 in the *Kiss1* promoter has a lower degree of methylation in *LSD1*^{-/-} females, which may contribute to an open chromatin conformation and enhanced *Kiss1* transcription.

Because of the likely involvement of the kisspeptin system in puberty, and the fact that Lomniczi et al. (2013) noted a pubertal increase in *Kiss1* gene expression in the ARC, the participation of *Eed* in affecting *Kiss1* at puberty was further investigated. Utilizing ChIP, Lomniczi et al. (2013) demonstrated that the EED protein dissociated from the *Kiss1* promoter in the ARC around the time of pubertal onset, and this was accompanied by an increase in histone 3 acetylation at lysine 9 and histone methylation at lysine 4, both of which are activational epigenetic marks. Next, by overexpressing *Eed* in the ARC region of peripubertal rats using a lentiviral vector, the authors showed that EED remained associated with the *Kiss1* promoter and that *Kiss1* expression was reduced by 50%, correlating with a reduction in GnRH secretion, a delay of puberty, disrupted estrous cyclicity, and decreased fecundity (Lomniczi et al., 2013). Though prominent, this effect was not as robust as when blocking DNA methyltransferases, likely because other repressive genes,

such as *Cbx7*, were unaffected by the *Eed* lentiviral vector. Overall, this study suggests that one pathway by which pubertal onset is regulated is via a DNA methylation-mediated reduction of PcG inhibition of the *Kiss1* gene in the ARC (i.e., a “repression of repressors”).

In addition to the recently published study above, preliminary data (conference proceedings) have addressed the involvement of LSD1 in the regulation of pubertal timing in mice, and, in particular, on *Kiss1* expression during puberty (Gill et al., 2012). As described earlier, LSD1 is a chromatin-remodeling enzyme (histone demethylase) that can regulate gene expression. More specifically, LSD1 acts to demethylate histones, and depending on which amino acid residue of the histone is affected, LSD1 can cause a repression or de-repression of gene expression. Unfortunately, LSD1 knockout mice are embryonic lethal and thus, cannot be studied for puberty; however, Gill and colleagues were able to examine mice heterozygous

for LSD1 (LSD1^{+/-}), which are viable. Interestingly, the researchers found that LSD1^{+/-} females have advanced vaginal opening and first estrus, two markers of puberty (Gill et al., 2012). Additionally, gonadotropin hormone levels were altered in LSD1^{+/-} female mice, with higher serum FSH detected in LSD1^{+/-} females compared to wild-type (WT) animals (Gill et al., 2012), suggesting that the reproductive axis was more stimulated in the former animals.

Gill and colleagues also quantified gene expression in the ARC (measured by qPCR) in prepubertal LSD1^{+/-} and WT females. Expression of both *Kiss1* and *Tac2* (another reproductive gene co-expressed in ARC *Kiss1* neurons) was higher in the ARC of peripubertal LSD1^{+/-} than peripubertal WT females, and LSD1 was found to be associated with the *Kiss1* promoter, as assessed via ChIP analysis of hypothalamic tissue (Gill et al., 2012). Looking in vitro, it was further shown in a *Kiss1*-expressing human breast cancer cell line (MDA-231) that knockdown of LSD1 expression by lentiviral shRNA transduction increased *Kiss1* expression, suggesting that LSD1 normally acts to repress this pubertal activator. Overall, though still preliminary, these new findings establish LSD1 as a possible contributing mechanism to pubertal progression, perhaps acting to alter *Kiss1* gene expression by lysine demethylation and/or acting on other key pubertal genes (Fig. 2B).

The interesting data discussed in this section highlight the importance of further investigations into the role of other chromatin remodeling factors in pubertal regulation. However, while both of the studies discussed above reported increases in pubertal *Kiss1* expression in the ARC, several other studies have failed to identify major pubertal *Kiss1* increases in this region (or only subtle increases). Thus, although epigenetic mechanisms may influence the *Kiss1* gene at the time of puberty, epigenetic modifications may also modulate other non-*Kiss1* pathways important for puberty.

6. Contributions of epigenetic mechanisms to sexually dimorphic *Kiss1* expression

Kiss1 in the AVPV/PeN is expressed to a greater degree in females than males, but it is unclear how the postnatal sex steroid milieu, namely, E₂ (aromatized from T), directs the sexual differentiation of the AVPV/PeN *Kiss1* system. A number of sex steroid-dependent mechanisms, such as differential neurogenesis, migration, epigenetics, and apoptosis, may be involved in the sexual differentiation and development of various neuronal populations (Forger, 2009; McCarthy et al., 2009; Murray et al., 2009; Simerly, 2002). Programmed cell death (apoptosis) is one of the primary mechanisms implicated in the sexual differentiation of the overall size and total cell number of the AVPV region, as well as other brain regions, such as the BNST (Forger et al., 2004; Gotsiridze et al., 2007; Krishnan et al., 2009). Most of these apoptosis-induced sex differences are dependent on the pro-apoptotic gene, *Bax* (Gotsiridze et al., 2007; Holmes et al., 2009; Krishnan et al., 2009), whose protein product, BAX, triggers caspase pathways that culminate in cell death (Adams and Cory, 2007). A previous study determined that the sex difference in total number of AVPV neurons, which is higher in females than males, is eliminated in *Bax* knockout mice (Forger et al., 2004), indicating that total cell number in the AVPV is sexually differentiated via *Bax*-dependent apoptotic mechanisms. Despite this finding, we recently found that sexual differentiation of *Kiss1* expression in the AVPV/PeN still occurs in *Bax*-knockout mice (Semaan et al., 2010). Thus, the *Kiss1* population is sexually differentiated either by other apoptotic pathways, such as tumor necrosis α -dependent or -independent mechanisms (as may be the case for AVPV GABA-ergic neurons (Krishnan et al., 2009)) or, by non-apoptosis related mechanisms, such as epigenetics.

Because we ruled out the involvement of BAX-dependent cell death in directing the sexual differentiation of AVPV/PeN *Kiss1* cells,

we recently proposed that the *Kiss1* sex difference is not induced by mechanisms that differentially affect the physical existence of cells, but rather by developmental mechanisms affecting transcriptional activity of the *Kiss1* gene (Poling and Kauffman, 2012b; Semaan and Kauffman, 2010; Semaan et al., 2012). In fact, epigenetic changes precipitated early in life by postnatal sex steroids are emerging as critical contributors to a number of sex differences in neuronal cell number and gene expression (Kurian et al., 2010; Matsuda et al., 2011; McCarthy et al., 2009; Murray et al., 2011; Nugent and McCarthy, 2010; Schwarz et al., 2010). As discussed above, histones are proteins that allow for the packaging of DNA into chromatin, and modification of histones, such as by acetylation, alters transcriptional activity (Goldberg et al., 2007). Histone acetylation, which is generally associated with increased transcriptional activity, has recently been implicated in the sex steroid-induced sexual differentiation of the BNST density and neuron number (Murray et al., 2009). For example, in mice, maintaining high levels of histone acetylation through the pharmacological inhibition of HDACs during the early postnatal critical period blocks the sexual differentiation of both the size of the BNST and vasopressin fiber projections (McCarthy et al., 2009; Murray et al., 2011). Postnatal HDAC inhibition also alters the sexual differentiation of male sexual behavior in rats and olfactory behavior in mice (Matsuda et al., 2011). Other recent studies have addressed how sexual differentiation is affected by DNA methylation, which occurs at individual CpG sites or on CpG sites comprising “CpG islands” in a gene or its promoter, and which is generally associated with the repression of gene expression. For example, studies have reported that the level of DNA methylation in the hypothalamus correlates with sexually differentiated expression of sex steroid receptor genes in this region (Kurian et al., 2010; Schwarz et al., 2010), and in the amygdala, which is a known sexually dimorphic brain region, the expression of DNA methyl transferase 3a (DNMT3a) in newborn rats was found to be sexually dimorphic (Kolodkin and Auger, 2011).

With the recent emerging evidence implicating the involvement of epigenetics in the sexual differentiation of various brain parameters, we investigated whether histone deacetylation and/or DNA methylation contributes to the AVPV/PeN *Kiss1* sex difference (Semaan et al., 2012). First, histone deacetylation was pharmacologically blocked during the postnatal critical period by administering an HDAC inhibitor (HDACi), valproic acid, to male and female mice on PND 1 and PND 2 and then analyzing AVPV/PeN *Kiss1* expression later in adulthood. This postnatal HDAC inhibition significantly increased the number of detectable *Kiss1* cells in the adult AVPV in each sex. Yet, sex differences in *Kiss1* expression and the number of detectable *Kiss1* cells in the AVPV/PeN were not eliminated (females still greater than males), indicating that histone deacetylation is not a stand-alone process for inducing the *Kiss1* sex difference (Semaan et al., 2012). However, the fact that overall *Kiss1* levels were higher in both sexes treated with HDACi suggests that the level of histone H3 acetylation during the critical period may be involved in modulating some aspect of the development of *Kiss1* neurons in the AVPV, perhaps by increasing the level of detectable *Kiss1* expression in each cell or by reducing apoptotic influences to thereby increase *Kiss1* cell survival. The critical period for sexual differentiation in mice lasts approximately a week, but HDACi treatment in our study was given only during the first two days of life (around the time of the endogenous T surge in newborn males), so it is still a possibility that histone deacetylation plays a role at later stages of the critical period. Despite this caveat, in the same study, we observed that sexual differentiation of the BNST was significantly disrupted by HDAC inhibition on just days 1 and 2 of life, indicating that this treatment paradigm is effective for modulating at least some sexually dimorphic phenotypes, just not AVPV/PeN *Kiss1* expression.

Because we did not find a definitive role for histone deacetylation in the sexual differentiation of AVPV/PeN *Kiss1* cells, we assessed whether the degree of DNA methylation of the *Kiss1* gene differed in the AVPV/PeN of males and females. Using microdissected tissue from the AVPV/PeN of adult males and females, we examined over 70 unique CpG sites in various regions of the *Kiss1* gene. Interestingly, we detected significant sex differences in the CpG methylation status of the *Kiss1* gene, mainly in the putative promoter region. In all cases, these sexually dimorphic *Kiss1* CpG sites were more methylated in females than males. Methylation of CpG sites can have multiple modes of affecting gene activity, but we tested if methyl-CpG binding protein-2 (MeCP2) was involved in the sex difference by assessing AVPV/PeN *Kiss1* levels in male and female *Mecp2* mutant mice. The AVPV/PeN sex difference was not eliminated in *Mecp2* mutant mice, suggesting that if DNA methylation is involved in the AVPV/PeN *Kiss1* sex difference, it might utilize the recruitment of other methyl binding proteins, such as MBD2, Kaiso, etc. Alternatively, it is also possible that differential methylation is affecting *Kiss1* gene expression via other mechanisms, such as by blocking the binding of transcriptional repressors which may regulate *Kiss1* expression (Semaan et al., 2012). Indeed, several of the identified sexually dimorphic *Kiss1* CpGs were in or near known binding sites for several transcriptional repressors, such as MZF1 and Gfi1.

Although these experiments increased our knowledge about the involvement (or lack thereof) of several epigenetic processes in the development of the AVPV/PeN *Kiss1* sex difference, more work is needed to elucidate the exact extent that certain processes, like DNA methylation, are involved. It is worth noting that, while intriguing, the *Kiss1* CpG methylation data was obtained on AVPV micropunches, which include a mixed population of both

kisspeptin and non-kisspeptin cells. Future work focusing specifically on individual kisspeptin cells will be important for verifying these findings and further testing the role of DNA methylation on *Kiss1* expression. Moreover, it is likely that the AVPV/PeN *Kiss1* sex difference is induced by several epigenetic processes affected by postnatal sex differences in the sex steroid milieu, and that these processes work in concert to cause an overall silencing of the *Kiss1* gene in males while allowing increased transcriptional activity in females (Fig. 3).

7. Effects of DNA methylation on human *KISS1* gene expression in vitro

Methylation of the human *KISS1* gene has also been investigated recently in relation to in vitro work in human cancer lines and as a potential prognostic and diagnostic indicator for clinical outcome in patients (Cebrian et al., 2011; Moya et al., 2012). *KISS1* mRNA expression and kisspeptin protein levels are often downregulated in cancer cells, and this low expression has now been shown to coincide with hypermethylation of a CpG island located near the transcription start site of the human *KISS1* gene (Cebrian et al., 2011; Moya et al., 2012). Interestingly, this particular CpG island is not present in the rodent *Kiss1* gene, and may represent a key species difference in how *Kiss1* expression can be regulated.

High levels of *KISS1* methylation have been frequently found in bladder cancer cells in vitro and bladder tumors. This high methylation pattern correlates with decreased *KISS1* expression in these cells and/or tissues (Cebrian et al., 2011). Additionally, high methylation levels and low *KISS1* expression were also associated with poor survival prognosis, thus suggesting that the level of *KISS1* methylation can potentially act as a biomarker for tumor

Fig. 3. Schematic depicting how epigenetics may influence the sexual differentiation of kisspeptin neurons in the AVPV/PeN. The purple cells depict detectable kisspeptin cells (i.e., cells in which *Kiss1* gene is expressed at sufficient levels), whereas the lighter cells with the dashed borders depict non-detectable kisspeptin cells which have little or no *Kiss1* expression. Evidence suggests that the total number of kisspeptin cells in the AVPV/PeN is likely not different between males and females. Rather, the expression of *Kiss1* by these cells differs between the sexes. This sex difference in *Kiss1* expression is directed by sex steroids during the critical postnatal period of development and may be induced, in part, by steroid-mediated changes in methylation of the *Kiss1* gene (Semaan et al., 2012). This particular modification may block the binding of transcriptional repressors to the *Kiss1* gene in females and/or work in concert with other epigenetic mechanisms to cause a silencing of the *Kiss1* gene in males, resulting in sexually dimorphic *Kiss1* expression.

progression. Similarly, a more recent study had comparable findings in colorectal cell lines and tumor tissues (Moya et al., 2012). In colorectal tumor tissues, *KISS1* hypermethylation was correlated with lower *KISS1* mRNA and kisspeptin protein levels. When treating immortalized cell lines in vitro with azacytidine (causing a loss of DNA methylation with multiple rounds of division), *KISS1* expression increased (Moya et al., 2012), indicated that in this experimental model, DNA methylation can affect *KISS1* expression. Diagnostically, the level of *KISS1* methylation in this study was an accurate predictor of disease specific survival, recurrence, and metastasis, and coupled with the use of common biomarkers for colorectal cancer, *KISS1* methylation status increased diagnostic accuracy (Moya et al., 2012). These initial investigations highlight the importance of epigenetic regulation of the human *KISS1* gene, and suggest further implications of epigenetic regulation relating to human health and disease.

8. Transcriptional regulation of *Kiss1* gene expression

Chromatin structure determines when and where certain transcription factors can bind. Thus, studies elucidating which transcription factors regulate *Kiss1* gene expression and where this regulation occurs within the *Kiss1* gene may provide insights into how certain regulatory regions of the *Kiss1* gene might be influenced by epigenetic contributions. However, at present, the involvement of specific transcription factors important for the regulation of *Kiss1* gene expression has not been well characterized. One study recently postulated that proteins thought to work upstream in the transcriptional network controlling female puberty may also control *Kiss1* gene expression (Mueller et al., 2011). These candidate transcription factors include CUX1, YY1, EAP1, and TTF1 (also known as Nkx2.1). Using double-immunofluorescence in rat brain tissue, Mueller and colleagues demonstrated that all of these transcription factors are present in ARC kisspeptin neurons, though not all kisspeptin neurons contain CUX1 (Mueller et al., 2011).

Intriguingly, most of these transcription factors are actually repressors of transcription. EAP1 has mainly repressive activities, and CUX1 can act as either a transcriptional repressor or activator, depending on how it is proteolytically processed (Sansregret and Nepveu, 2008). YY1 is a member of the PcG silencing complex and recruits other repressive proteins (Wilkinson et al., 2006; Woo et al., 2010). TTF1 is important for the development of the diencephalon (Kimura et al., 1996), and appears to be critical for proper pubertal development (Mastronardi et al., 2006). Mueller and colleagues found that all of these transcription factors can be recruited to the *Kiss1* promoter. These investigators carried out a number of in vitro studies testing the function of these transcription factors on *Kiss1* gene expression. However, most of their repressive or activational effects in vitro on *Kiss1* gene activity only occurred in non-neuronal cells (immortalized fibroblasts) and not in the GT1-7 hypothalamic cell line (Mueller et al., 2011). For example, YY1 had a very strong repressive role on *Kiss1* in non-neuronal cells, but did not have an effect in GT1-7 cells (neuronal cell line expressing GnRH). Similarly, EAP1 also had a repressive effect on *Kiss1*, but it was only consistently observed in non-neuronal cells. TTF1, on the other hand, was stimulatory, but was still only able to trans-activate the proximal *Kiss1* promoter in non-neuronal cells. Conversely, the longer isoform (p200) of CUX1 was repressive to the proximal promoter segment of *Kiss1* in neuronal cells, but was activational in non-neuronal cells (Mueller et al., 2011).

Overall, these studies provide evidence that *Kiss1* gene expression may be heavily regulated by transcriptional repressors, but more research will be needed to identify the role of these repressors at the physiological level, specifically in hypothalamic kisspeptin neurons during puberty. For example, these repressors may act

differentially on *Kiss1* gene expression in the AVPV/PeN and ARC populations, especially before and during puberty. If such studies confirm the actions of these transcription factors on the *Kiss1* gene, it will be critical to then see if epigenetic processes influence these factors' regulation of *Kiss1* expression.

9. Conclusions

Although the newest studies on epigenetics and *Kiss1* expression have increased our understanding of the different mechanisms involved in the regulation and development of *Kiss1* gene expression, there are important questions that have yet to be resolved. Such issues include defining the specific timing when these epigenetic mechanisms are activated (or deactivated) and determining how they all interact for the proper development and regulation of *Kiss1* gene expression. Future studies will likely utilize transgenic and knockout animals, with the goal of teasing apart the specific roles of all of these various epigenetic modifications in *Kiss1* neurons specifically, especially during periods of sexual differentiation and pubertal maturation. Similarly, it will be important to further define the important regulatory regions of the *Kiss1* gene, and identify critical transcription factors that regulate *Kiss1*, in order to better elucidate the complex mechanisms involved in its regulation. Future studies are also needed to further dissect the effects of sex steroids on different epigenetic mechanisms, as steroid hormones are involved in both permanently sexually differentiating the *Kiss1* gene in the AVPV/PeN early in development, as well as transiently regulating *Kiss1* gene expression in a differential manner in the ARC and AVPV/PeN in adulthood.

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