Epigenetic control of female puberty

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The timing of puberty is controlled by many genes. The elements coordinating this process have not, however, been identified. Here we show that an epigenetic mechanism of transcriptional repression times the initiation of female puberty in rats. We identify silencers of the Polycomb group (PcG) as principal contributors to this mechanism and show that PcG proteins repress Kiss1, a puberty-activating gene. Hypothalamic expression of two key PcG genes, Eed and Cbx7, decreased and methylation of their promoters increased before puberty. Inhibiting DNA methylation blocked both events and resulted in pubertal failure. The pubertal increase in Kiss1 expression was accompanied by EED loss from the Kiss1 promoter and enrichment of histone H3 modifications associated with gene activation. Preventing the eviction of EED from the Kiss1 promoter disrupted pulsatile gonadotropin-releasing hormone release, delayed puberty and compromised fecundity. Our results identify epigenetic silencing as a mechanism underlying the neuroendocrine control of female puberty.

Much has been learned in recent years about the neuroendocrine mechanisms controlling the initiation of female reproductive function. It requires changes in the release of gonadotropin-releasing hormone (GnRH) from neurosecretory neurons mostly located in the medial basal hypothalamus of primates and in the preoptic region of rodents1,2. These changes are, in turn, determined by modifications in trans-synaptic3 and glial4 inputs to the GnRH neuronal network. Whereas the trans-synaptic changes involve an increase in excitatory inputs and a reduction in inhibitory influences4, the glial component is predominantly facilitatory and is exerted by both growth factors and small molecules that directly or indirectly stimulate GnRH secretion4. The direct excitatory trans-synaptic regulation of GnRH secretion is provided by at least three different neuronal subsets: kisspeptin neurons acting via the G protein–coupled receptor KISS1R (also known as GPR54)5, glutamatergic neurons acting mostly via AMPA receptors6,7 but also via NMDA receptors7,8, and GABA acting via ionotropic GABAA receptors9. The inhibitory counterpart of this circuitry depends principally on GABAergic neurons acting via GABAB metabotropic receptors9 but also on opiatergic neurons that employ different peptides and a variety of different receptors for inhibitory neurotransmission (reviewed in ref. 1).

As predicted by the complexity of this cellular network, several reports have suggested that no isolated pathway or cellular subset is solely responsible for the neuroendocrine control of puberty10–12. Instead, initiation of this process may require regulatory gene networks controlled by a handful of upstream genes10. Some of these central nodes have been identified, including the Pou-domain gene Pou2f2 (also known as Oct2), the homeodomain gene Nkx2-1 (Tf-1), and a zinc finger–containing gene termed Irf2bp1 (interferon regulatory factor 2 binding protein-like; also known as Eap1, for Enhanced At Puberty 1)13. Although monogenic mutations such as those affecting GNRHR14, KISS1R (GPR54)15,16, Kiss1 (ref. 17), TAC3 and TACR3 (ref. 18) result in pubertal failure, it does not appear that these are the only puberty-relevant genes, as genome-wide association studies have shown that variants of more than 30 genes are associated with the age of menarche in humans19.

It is thus apparent that the genetic underpinnings of puberty are multigenic, but this realization does not explain how inherited, permanent changes in DNA sequence can regulate gene expression dynamically while also imposing an encompassing level of coordination and transcriptional plasticity on the gene networks involved. Here we develop the concept that a biological regulatory system that meets these requirements is epigenetics. Our results provide proof of principle for the view that the timing of female puberty is under the regulatory control of an epigenetic mechanism of transcriptional repression. We identify the Polycomb group (PcG) of transcriptional silencers20 as integral parts of this repressive mechanism and implicate two PcG genes (Cbx7 and Eed) as encoding core components of the PcG complex operating in the prepubertal hypothalamus. Using the Kiss1 gene as a prototype of a gene whose products are directly involved in controlling GnRH output21, we provide evidence that the PcG complex represses the advent of reproductive maturity by targeting downstream genes involved in the stimulatory control of GnRH secretion at puberty.

RESULTS

Inhibition of DNA methylation results in pubertal failure

To gain insights into the potential contribution of DNA methylation to the regulation of puberty, we inhibited DNA methylation by treatment with 5-azacytidine (AzA), a well-established DNA methyltransferase...
(DNMT) inhibitor\(^{22,23}\). The treatment (2 mg per kilogram body weight per day, intraperitoneally) was initiated on postnatal day (P) 22, which in the rat corresponds to the initiation of the early juvenile phase of pubertal development\(^2\). We first evaluated the effect of Aza on the timing of puberty and estrous cyclicity by continuing the treatment until P44; that is, nearly 2 weeks after all control rats (injected with saline solution) had reached puberty. In all subsequent studies, the rats were treated only for the duration of the juvenile period; that is, from P22 to P28. Rats subjected to long-term Aza treatment had delayed vaginal opening (Fig. 1a; mean age at vaginal opening: control, 31.33 ± 0.21 d, \(n = 6\); versus Aza, 36.67 ± 0.67 d; \(t = -7.628, P < 0.001\), Student’s \(t\) test); failed to reach puberty, as assessed by the lack of ovulation; and showed no estrous cyclicity, as determined by daily vaginal lavages after vaginal opening (Fig. 1b). These alterations did not appear to result from a nonspecific effect of Aza, because the rats treated with the inhibitor weighed substantially more (20 g) than control rats at the time of vaginal opening and had not achieved puberty by the end of the experiment even though they weighed 35 g more than the weight of the inhibitor weighed substantially more (20 g) than control rats at the end of the experiment even though they weighed 35 g more than the weight of the control rats (control, 20 g; Aza, 40 g; \(t = 6.48, P < 0.001\), Student’s \(t\) test), and Aza-treated rats had antral follicles but no corpora lutea, indicating that they had not ovulated and, consequently, that puberty had failed to occur.

To determine where Aza may be acting to prevent the advent of puberty, we first examined the competence of the ovary to respond to gonadotropins with estradiol production. We treated rats with Aza from P22 to P28, administered a single subcutaneous injection of pregnant mare serum gonadotropin (PMSG, \(8\) IU per rat) on P26 and collected trunk blood for estradiol measurement on P28. The Aza treatment did not inhibit, and even enhanced, the estradiol response of the ovary to PMSG (Fig. 2a). This outcome suggested that the delay in puberty was due to a central or pituitary, instead of an ovarian, defect.

Consistent with this interpretation, basal plasma luteinizing hormone was lower in P28 Aza-treated rats than vehicle-treated controls (control, 1.89 ± 0.16 ng ml^{-1}, \(n = 19\); Aza, 0.55 ± 0.16 ng ml^{-1}, \(n = 23\); \(t = 3.23, P = 0.002\), Student’s \(t\) test), and Aza-treated rats had a greatly diminished luteinizing hormone response to ovarectomy, performed on P24 and assessed on P28 (control, 52.56 ± 6.93 ng ml^{-1}, \(n = 6\); versus Aza, 7.43 ± 2.76 ng ml^{-1}, \(n = 6\); \(t = 6.48, P < 0.001\), Student’s \(t\) test). Despite this deficiency, the pituitary response to \textit{in vivo} administration of GnRH on P28 was normal in Aza-treated rats (Fig. 2b), indicating the absence of a pituitary defect. To evaluate the ability of GnRH neurons to respond to a physiological neuroendocrine stimulus, we exposed medial basal hypothalamic (MBH) fragments from P28 rats, which contain the median eminence–arcuate nucleus (ME-ARC) region, to kisspeptin, a potent GnRH secretagogue\(^{24}\). The ME-ARC from Aza-treated rats responded to kisspeptin with significantly more GnRH release than vehicle-treated controls (Fig. 2c), suggesting cellular hyper-responsiveness presumably due to a deficiency in endogenous kisspeptin production.

\(^{21}\) At the time when vaginal opening has occurred in all saline-injected controls (P32), vaginal patency was not apparent in any of the Aza-treated rats.

\(^{22}\) PMSG, pregnant mare serum gonadotropin.

\(^{23}\) Student’s \(t\) test.

\(^{24}\) In vivo administration of GnRH on P28 was normal in Aza-treated rats (Fig. 2b), indicating the absence of a pituitary defect.

\(^{25}\) The ME-ARC from Aza-treated rats responded to kisspeptin with significantly more GnRH release than vehicle-treated controls (Fig. 2c), suggesting cellular hyper-responsiveness presumably due to a deficiency in endogenous kisspeptin production.
Thus, the delay of Aza action is indeed to be attributed to reduced DNA methylation in the hypothalamus. We measure global DNA methylation in the MBH and cerebral cortex at two intervals (1 and 7 d) after starting Aza treatment. At both time points, DNA methylation was significantly reduced in the MBH but not in the cerebral cortex (CTX) as compared with diluent-treated controls (in MBH: 1 d of treatment, 100 or 500 nM of the 10-amino-acid peptide kisspeptin-10 (Kp) (interaction between treatment group and Kp dose), 3,31 = 5.93, P = 0.007, two-way repeated-measures ANOVA, n = 6 rats per group). (c) Hyper-response of the GnRH system to kisspeptin, as determined by the in vitro release of GnRH from ME-ARC fragments (derived from P28 rats) incubated with either 100 or 500 nM of the 10-amino-acid peptide kisspeptin-10 (Kp) (interaction between treatment group and Kp dose, F3,47 = 0.928, P = 0.441; 7 d of treatment t = –0.928, P = 0.375; Student’s t test, n = 6 rats per group). Means ± s.e.m. throughout. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 2  In vivo inhibition of DNA methylation prevents puberty by disrupting developmental events upstream from the GnRH-pituitary-ovarian axis. (a) Treatment with Aza in fact augments the estradiol response of the ovary to stimulation with PMSG (F2,31 = 47.81, P < 0.001, one-way ANOVA followed by Student-Newman-Keuls (SNK) multiple comparison test, n = 8 rats per group). (b) GnRH (6.5 μg kg−1 intraperitoneally) injection increases plasma luteinizing hormone (LH) similarly in vehicle-treated control and Aza-treated rats (between time points within each treatment, F2,31 = 277.23, P < 0.001, but between treatments, F1,16 = 0.46, P = 0.52, with no significant interaction between time points and treatments, F2,47 = 0.56, P = 0.58, two-way repeated-measures ANOVA, n = 8 rats per group). (c) Hyper-response of the GnRH system to kisspeptin, as determined by the in vitro release of GnRH from ME-ARC fragments (derived from P28 rats) incubated with either 100 or 500 nM of the 10-amino-acid peptide kisspeptin-10 (Kp) (interaction between treatment group and Kp dose, F3,47 = 0.928, P = 0.441; 7 d of treatment t = –0.928, P = 0.375; Student’s t test, n = 6 rats per group). Means ± s.e.m. throughout. *P < 0.05; **P < 0.01; ***P < 0.001.

Because these results implied a hypothalamic site of Aza action, we carried out a study to verify the ability of Aza to reduce DNA methylation in the hypothalamus. We measure global DNA methylation in the MBH and cerebral cortex at two intervals (1 and 7 d) after starting Aza treatment. At both time points, DNA methylation was significantly reduced in the MBH but not in the cerebral cortex (Fig. 2d). This difference may be due to the lack of a fully functional blood–brain barrier in the MBH, a feature that allows transfer of macromolecules from the bloodstream to the hypothalamic parenchyma25,26.

To determine whether Aza may have delayed puberty by affecting hormonal systems other than that controlling the hypothalamic-pituitary-ovarian axis, we measured serum prolactin (PRL) because PRL is produced independently from luteinizing hormone, delays puberty when secreted at subnormal levels and disrupts estrus cyclic-... (continued)

A repressive complex is silenced at puberty

To search for potential repressor genes that may become more methylated in the hypothalamus at puberty, we used DNA methylation arrays to interrogate the MBH at different pubertal stages (early juvenile, late juvenile and late proestrus). The late proestrus phase of puberty corresponds to the day of the first preovulatory surge of gonadotropins, which in the rat can be considered as mid-puberty2. We observed (see Supplementary Fig. 3) that genes with a pattern of changing methylation at either the late juvenile or late proestrus phases of puberty were functionally enriched for a cluster of chromatin/histone modification terms. Several of these genes are components of a common regulatory pathway as they were either members of the PcG silencing complex (Cbx7, Cbx8, Phc3, Yy1 and Rnf2 (also known as Ring1B)) or encoded proteins that interact with PcG proteins (Rybp, Csnk2b, Kdm2b). With the exception of Rnf2, all of these genes exhibited a general pattern of DNA methylation decrease in the hypothalamus of Aza-treated rats.
increased promoter methylation at puberty (Supplementary Fig. 2), suggesting that the PcG silencing complex may undergo functionally important epigenetic changes with the advent of puberty.

The PcG silencing complex is considered a master regulator of genomic programs because it acts at different stages of development to define which sets of genes are active and which ones are quiescent\(^{20,28}\). The PcG system is composed of three repressive complexes (PRC1, PRC2 and PhoRC) working together to silence genes\(^{20,28}\). The PRC1 complex (Supplementary Fig. 3) includes a group of proteins termed CBX because they contain a highly conserved chromodomain (CBX) at their amino terminus\(^{29}\). The mammalian homologs of Drosophila polycomb proteins are CBX2, CBX4, CBX6, CBX7 and CBX8 (ref. 28).

In different cells, the PRC1 complex may contain different CBX proteins\(^{30}\). The PRC2 complex includes four core subunits: enhancer of Zeste (EZH1, EZH2), suppressor of Zeste (SUZ12) and the WD40 domain proteins EED and P55 (RBP4 and RBP7)\(^{28}\) (Supplementary Fig. 3). PhoRC, the third PcG complex in Drosophila, contains two proteins, Pho and its homolog Phol, which bind directly to DNA\(^{28}\). In mammals, these proteins are encoded by the Yy1 gene (Supplementary Fig. 3), which has both repressive and activating functions\(^{31}\).

To determine whether the changes in promoter DNA methylation of PcG genes suggested by the arrays are accompanied by altered gene expression, we focused our attention on the PcG complex and measured by quantitative (q) PCR the mRNA abundance of most members of this complex in the MBH at the time of puberty. Expression of only two PcG genes required for appropriate PcG function\(^{29,32}\), the PRC1 member Cbx7 and the PRC2 member Eed (not represented in the DNA arrays), decreased at the late juvenile stage; that is, at the time when puberty is initiated in the female rat\(^3\). This reduction was maintained throughout puberty (Fig. 3a), seemingly unaffected by the peripubertal increase in serum estradiol (Fig. 3b), but was prevented by the administration of Aza (Fig. 3a). Among the other members of the PcG complex examined, only Yy1 mRNA decreased significantly (\(P < 0.05\)) at puberty, but this decrease occurred much more gradually (Supplementary Fig. 3). No reduction in mRNA of any member of the PcG complex, including Cbx7 and Eed, was seen in the preoptic area during puberty (Supplementary Fig. 3), indicating that the pubertal decrease in Cbx7 and Eed expression was MBH-specific.

The advent of puberty was associated with increased promoter methylation of both Cbx7 and Eed (Fig. 3c), and this change was prevented by Aza (Fig. 3c). The regions examined for changes in

**Supplementary Figure 2**

**Figure 4** The Cbx7 and Eed genes are expressed in kisspeptin neurons of the ARC. (a) Double fluorescence in situ hybridization showing the presence of Cbx7 and Eed mRNA (green) in Kiss1 mRNA (red)-containing neurons. Arrows point to double-positive neurons; asterisks denote Kiss1 mRNA–positive neurons without detectable Cbx7 or Eed mRNA. Scale bars, 20 μm. (b) Single-cell PCR of ten eGFP-tagged mouse kisspeptin neurons\(^{30}\) (lanes 1–10) demonstrating the presence of Cbx7 and Eed mRNAs in these cells. MBH tissue was used as positive control (+C); as negative controls, no reverse transcription (–C) and water input.

**Figure 5** Increased Kiss1 expression in the MBH at the initiation of puberty is accompanied by eviction of EED from the Kiss1 promoter and changes in associated repressive and activating histone modifications, without changes in DNA methylation. AU, arbitrary units. (a) Kiss1 mRNA abundance increases in the MBH at between early juvenile (EJ) and late juvenile (LJ) stages, and this increase is prevented by inhibition of DNA methylation (Fig. 2, 1, 7.11, 0.005). (b) Kiss1 mRNA abundance does not change in the MBH at the onset of puberty, but it is elevated after inhibition of DNA methylation (Fig. 2, 4.78, 0.021). (c) Methylation of the Kiss1 promoter in the MBH does not change at puberty, but it is diminished by inhibition of DNA methylation (Fig. 2, 13.93, 0.001). (d) EED is evicted from the Kiss1 promoter at the initiation of puberty, and this eviction fails to occur in Aza-treated rats (Fig. 2, 17.98, 0.001). (e) The association of H3K27me3 to the Kiss1 promoter does not decrease at LJ and is not affected by Aza (Fig. 2, 3.45, 0.037 only when the β-gal group is included). (f) The abundance of H3K4me3 decreases at LJ, and the increase is prevented by inhibition of de novo DNA methylation (Fig. 2, 76.09, 0.001). (g) The association of H3K9,14ac also increases at LJ, and this increase fails to occur in Aza-treated rats (Fig. 2, 12.43, 0.001). For all panels except c, n = 6–8 rats per group; for c, n = 4 or 5. Means ± s.e.m. throughout. *P < 0.05, ***P < 0.001 versus EJ; in all cases, one-way ANOVA was followed by the SNK multiple comparison test for unequal replications. Antibodies to β-galactosidase (β-Gal) (a protein not expressed in the rat) served as a negative control; dotted lines indicate minimum sensitivity of the technique.
methylation are shown in Supplementary Figure 4. The increase in DNA methylation did not result from changes in circulating estradiol amounts, because it was distinct at late juvenile, when plasma estradiol is still low, and remained unaltered on the day of the first preovulatory surge of gonadotropins (Fig. 3c), when plasma estradiol is much higher (Fig. 3b). Altogether, these results suggest that the onset of female puberty is accompanied by active epigenetic repression of the PcG silencing system in the MBH of female rats.

The Kiss1 gene is a downstream target of PcG repression

In all mammalian species so far studied, the first neuroendocrine manifestation of puberty is a diurnal increase in pulsatile GnRH and hence luteinizing hormone release (reviewed in ref. 2). This mode of GnRH secretion has been postulated to be driven by a subset of ARC neurons, called KNDy neurons33,34 because they produce kisspeptin, NKB and dynorphin33. Because kisspeptin and NKB work coordinately in the population of KNDy neurons to stimulate GnRH secretion, their encoding genes (Kiss1 and Tac2) can be considered components of a distinct class of puberty-activating genes. Using Kiss1 as a prototype of this class, we carried out studies to determine whether Cbx7 and Eed are expressed in kisspeptin neurons of the ARC. Double fluorescence in situ hybridization (Fig. 4a) and single-cell PCR (Fig. 4b) of eGFP-tagged mouse kisspeptin neurons35 showed that these neurons contained both Eed and Cbx7 mRNAs.

Measurement of the mRNAs encoding kisspeptin and its receptor Kiss1R in the MBH demonstrated that Kiss1 mRNA abundance increased in this brain region between early juvenile and late juvenile stages and that inhibition of DNA methylation prevented this change (Fig. 5a). In contrast, Kiss1r mRNA abundance remained unaltered in late juvenile rats as compared with early juvenile but was significantly increased by the inhibition of DNA methylation (Fig. 5b). These results suggest that the hyper-responsiveness of the GnRH neuronal network to kisspeptin seen in Aza-treated rats may be related, at least in part, to a reduced endogenous production of kisspeptin in the presence of upregulation of its receptor Kiss1R. Methylation of the Kiss1 promoter remained unchanged in the MBH of late juvenile rats as compared with early juvenile (Fig. 5c), indicating that the increase in Kiss1 mRNA observed at the end of juvenile development is not caused by alterations in promoter methylation. Although Aza decreased Kiss1 promoter methylation (Fig. 5c), it obliterated the pubertal increase in Kiss1 mRNA, suggesting that inhibition of DNA methylation prevents the pubertal increase in Kiss1 expression by mechanisms other than changes in Kiss1 promoter methylation.

Because MBH expression of both Cbx7 and Eed appeared to be DNA methylation dependent and because EED is essential for PcG action32, we selected Eed for further analysis. In silico analysis of the Kiss1 promoter (Supplementary Fig. 5a) demonstrated that it contained several motifs found to be present in PcG target genes, including the core motif for YY1 binding (CCAT), the GAf (GAGAG) and extended MPho (CNGCCATNDNNND) motifs36, two BMI1 binding motifs (CCTTCC and GGNNNGNG) previously reported37, and the binding motif for HOTAIR, a long noncoding RNA, recently shown to serve as an anchor for PcG binding to gene promoters38. To determine whether these motifs have biological significance, we performed gene promoter assays. EED indeed repressed Kiss1 promoter activity, and this repressive effect was enhanced by YY1 (Supplementary Fig. 5b). Next, we carried out chromatin immunoprecipitation (ChIP) assays to determine (i) whether EED is recruited to the Kiss1 promoter in the MBH and (ii) whether this relationship changes during the onset of puberty. EED protein was associated with the Kiss1 promoter at the early juvenile stage, and this association decreased at the late juvenile stage, the time of initiation of puberty (Fig. 5d). Consistent with the notion that inhibition of DNA methylation leads to increased expression of PcG genes, which then repress downstream targets genes, the pubertal loss of EED association to the Kiss1 promoter did not occur in Aza-treated rats (Fig. 5d). The chromatin status of the Kiss1 promoter also changed at the time of puberty. Whereas the content of histone H3 trimethylated on Lys27 (H3K27me3), a PcG-dependent repressive histone modification39,40, did not decrease significantly in late juvenile rats (Fig. 5e), the abundance of two activating histone H3 modifications, trimethylation on Lys4 (H3K4me3) and acetylation on Lys9 and Lys14 (H3K9,14ac)39,41,42, increased markedly at this time (Fig. 5f,g). Treatment with Aza, which prevented the eviction of EED from the Kiss1 promoter at the late juvenile stage, also prevented the late juvenile increase in both H3K4me3 and H3K9,14ac abundance (Fig. 5f,g). To determine whether the chromatin landscape of the Kiss1 promoter continues to change as the pubertal process unfolds, we measured both H3K27me3 and its opposing counterpart H3K4me3 (ref. 43) at late proestrus, when the postovulatory surge of gonadotropins takes place, and found a significant (P < 0.05) decrease in H3K27me3,
accompanied by persistently elevated H3K4me3 (Supplementary Fig. 6a). Altogether, these results are compatible with the notion that a repressive PcG-dependent influence on the Kiss1 gene is lifted at the onset of puberty and the status of the associated chromatin shifts from an inhibitory to an activating configuration, leading to activation of the Kiss1 gene (Supplementary Fig. 6b).

Overexpressing EED compromises reproductive capacity

If PcG proteins influence neuroendocrine control of female puberty by repressing the Kiss1 gene in the ARC, then preventing the pubertal decrease in PcG gene expression that occurs in this hypothalamic region at the onset of puberty would be expected to delay puberty. Because Eed is required for the silencing activity of the PcG complex\(^1\), we chose to overexpress EED in the ARC of immature female rats. We cloned the coding region of rat Eed tagged with a hemagglutinin (HA) epitope into a lentiviral vector (LV) that also expresses eGFP (Supplementary Fig. 7a). After confirming the production of the HA-tagged protein by western blot (Supplementary Fig. 7b), we stereotaxically delivered this construct (termed LV-EED) bilaterally into the hypothalamus of P26 female rats, targeting the ARC. Control rats were injected with a construct expressing only eGFP (LV-eGFP). We used immunohistofluorescence analysis of the sites of injection using antibodies to eGFP to identify the transduced cells (Fig. 6a). Kisspeptin neurons, also identified by immunohistofluorescence, were one of the cell populations transduced by the virus (Fig. 6b,c). ChIP analysis of DNA extracted from microdissected ARC tissue containing the transduced cells revealed that the LV-produced EED-HA protein had been recruited to the Kiss1 promoter (Fig. 6d). The number of detectable immunopositive kisspeptin cells per section decreased 25% in LV-EED-injected rats (LV-GFP, 21.4 ± 1.98, n = 31; LV-EED, 16.4 ± 1.7, n = 31; Supplementary Fig. 7b). We used immunohistofluorescence analysis of the sites of injection

![Figure 7](https://example.com/image7.png)

**Figure 7** EED delivered to the ARC of immature female rats inhibits GnRH pulse frequency without changing pulse amplitude. (a) Individual profiles of GnRH release from LV-GFP- and LV-EED-injected rats, n = 6 rats per group. Arrows indicate individual pulses. (b) Left panel: GnRH pulse frequency was significantly (t = 25.5, P = 0.026, Mann-Whitney rank sum test) reduced in LV-EED-injected rats, as determined by an increase in interpulse interval. Middle panel: pulse amplitude remained unchanged (t = -0.307, P = 0.765, Student’s t test). Right panel: total GnRH output per 4-h incubation was significantly (t = 2.61, P = 0.026, Student’s t-test) reduced in the LV-EED treated rats. (c) Eed mRNA content was fourfold higher (t = -5.77, P < 0.001) and Kiss1 mRNA content 50% lower (t = 3.17, P = 0.01) in LV-EED-injected rats than in LV-GFP-injected (Student’s t-test). In all cases, n = 6 rats per group. Means ± s.e.m.
reached the periovulatory stage (Fig. 8c). In contrast, LV-GFP-injected rats had an abundance of corpora lutea, indicating repeated ovulations (Fig. 8c). In a third experiment, we delivered the LV-GFP and LV-EED constructs to the ARC of P22 rats and, after all rats in the LV-GFP-injected group showed three complete estrous cycles, exposed each rat to a fertile male for 5 d. Rats in which the LV-EED construct was correctly targeted to the ARC had fewer pups or failed to deliver a litter on exposure to a fertile male (Supplementary Table 3), in comparison to the >90% fertility observed in LV-GFP-injected controls (Fig. 8d). Thus, preventing the reduction in Eed expression that occurs in the ARC at the onset of puberty compromises GnRH pulsatile release, delays the pubertal process, disrupts estrous cyclicity, reduces ovulation, and decreases fecundity. Altogether, these results are consistent with the interpretation that the onset of female puberty is controlled by a PcG-dependent repressive mechanism involving silencing of the Kiss1 gene in kisspeptin neurons of the MBH.

DISCUSSION

The potential contribution of epigenetics to the regulation of puberty has, to our knowledge, never been addressed. In the present report, we provide evidence that an epigenetic mechanism of transcriptional repression, operating in the neuroendocrine brain, influences the timing of female puberty. Our results identify the PcG system of transcriptional silencing20,28 as a central element of this repressive mechanism. Hypothalamic expression of Cbx7 and Eed, two PcG genes required for PcG action29,32, decreased before the onset of puberty, and this change was associated with increased DNA methylation of their 5′ flanking regions. Conversely, pharmacological inhibition of DNA methylation prevented the pubertal increase in Eed and Cbx7 DNA methylation, reversed the low peripubertal Eed and Cbx7 mRNA levels to elevated early-juvenile values, and delayed puberty. This delay was not due to nonspecific or toxic effect of the inhibitor, because the rats failed to reach puberty despite a body weight much greater than that attained by control rats at puberty. Moreover, it was not caused by changes in the secretion of two hormones, PRL and corticosterone, that in deficiency (PRL) or excess (corticosterone) have been shown to delay puberty in the rat (reviewed in ref. 2). Within the hypothalamic-pituitary-ovarian axis, inhibition of DNA methylation did not affect the capacity of the ovary to respond to gonadotropin stimulation with estrogen release and did not alter the pituitary gonadotropin response to GnRH, suggesting a central site of action. Direct assessment of the GnRH response to kisspeptin, a principal GnRH secretagogue34, revealed that GnRH neurons of Aza-treated rats were hyper-responsive, instead of unresponsive, to kisspeptin. Although Aza, like other DNMT inhibitors, may also act through mechanisms other than DNA methylation45,46, our results are consistent with the interpretation that pharmacological inhibition of DNA methylation prevents a methylation event scheduled to occur at the onset of puberty. Without ruling out GnRH neurons as direct targets of epigenetic control47, our results suggest that (i) the pubertal delay caused by inhibition of DNA methylation involves cellular subsets functionally connected to the GnRH neuronal network and that (ii) the deficit may result from the activation of repressive genes whose expression would normally decrease at puberty. By inference, these repressors would be expected to negatively control the expression of downstream genes that must be activated for puberty to occur. A search for such repressors using DNA methylation arrays suggested that the initiation of puberty was accompanied by changing methylation of the promoters of several members of the PcG repressive complex and genes encoding proteins that interact with the PcG system. If this change predicts opposite changes in gene expression, one would expect to find decreased hypothalamic expression of PcG genes either during puberty or immediately before it. Measuring the expression of most PcG components in the MBH by qPCR demonstrated an early decrease in Cbx7 and Eed mRNA abundance before the initiation of puberty and a drop in Yyl1 expression at mid-puberty. EED is a PRC2 component required for PcG action32. The decrease in Cbx7 and Eed expression occurred independently from changes in ovarian estrogen output, as it was essentially complete before the pubertal increase in circulating estrogen.
Because the kisspeptin–KISS1R system is critical for both puberty and adult reproductive function\textsuperscript{5,16}, the Kiss1 gene can be considered a prototype of the class of genes that need to be activated for puberty to occur. Accordingly, we used the Kiss1 gene to test the hypothesis that these puberty-activating genes may be subjected to PcG repressive control. The increase in Kiss1 mRNA abundance that occurs in the hypothalamus at the time of puberty was prevented, instead of enhanced, by inhibition of DNA methylation, suggesting that a secondary mechanism set in motion by the loss of DNA methylation is responsible for the reduction in Kiss1 expression. A notable component of this mechanism appears to be the PcG silencing complex, as the prepubertal association of EED to the Kiss1 promoter, which diminished at the onset of puberty, was prevented by inhibition of DNA methylation.

PcG-mediated gene silencing requires H3K27me3, a modification catalyzed by PRC2. H3K27me3 then provides a docking site for the CBX components of PRC1 to form a repressive complex\textsuperscript{20,28}. In turn, YY1 recruits PRC2 and PRC1 proteins, in addition to H3K27me3, to gene promoters to enhance transcriptional silencing\textsuperscript{32}. The eviction of EED from the Kiss1 promoter at the onset of the pubertal process would predict a concomitant loss of H3K27me3 at this time. Instead, H3K27me3 content decreased at late proestrus; that is, by mid-puberty. Contrasting with this protracted pattern of change, the abundance of H3K4me3 and H3K9/14ac, two histone marks associated with gene activation\textsuperscript{39,41}, increased markedly at the late juvenile stage; that is, at the initiation of puberty. Because H3K4me3 is a histone mark that opposes the repressive actions of H3K27me3 (ref. 43), we examined the association of H3K4me3 to the Kiss1 promoter at mid-puberty and found it to remain as elevated as at the late juvenile stage. This developmental profile is consistent with the pattern of bivalent association observed for H3K27me3 and H3K4me3 in the promoters of genes mildly derepressed during development\textsuperscript{48}. The evolving presence of both marks on the Kiss1 promoter at puberty is also consistent with the concept of “bivalent” domains\textsuperscript{49}; that is, the simultaneous presence of repressive and activating histone modifications\textsuperscript{48,49} in the regulatory regions of genes thought to be poised for activation in response to developmental cues\textsuperscript{50}. Notably, the pubertal increase in the association of activating histone marks to the Kiss1 promoter failed to occur in Aza-treated rats. Because the pubertal EED eviction also fails to occur in these animals, the simplest explanation is that persistent EED occupancy diminishes accessibility of activating histone marks to the Kiss1 promoter.

Directly supporting the overall validity of a PcG-dependent repressive mechanism holding in check the initiation of puberty is the pubertal delay observed when the decline in hypothalamic Eed expression that occurs during normal puberty is prevented by means of targeted lentivirus-mediated gene delivery. Overexpression of Eed in the ARC of the hypothalamus, which contains the KNDy neurons required for pulsatile GnRH release\textsuperscript{34}, reduced the number of neurons expressing detectable immunoreactive kisspeptin, the content of immunoreactive kisspeptin per cell and the abundance of Kiss1 mRNA in the ARC. And notably, it reduced pulsatile GnRH release, delayed puberty, and disrupted estrous cyclicity. Although the rats receiving lentiviral particles carrying the EED gene were still able to ovulate, their estrus cycle profiles suggested that they were ovulating sporadically. This inference is supported by the finding that these females delivered an average of 2 pups when exposed to a fertile male, as compared with 12 pups delivered by rats receiving either a control virus or a virus expressing EED but targeted outside the ARC.

By showing that the neuroendocrine control of female puberty involves the participation of a repressive mechanism of epigenetic regulation, our results provide insight into the integrative mechanisms used by the neuroendocrine brain to control the initiation of mammalian puberty. As such, they are consistent with the concept that the pubertal process depends not only on genetic determinants, but also on developmentally regulated changes in epigenetic information. They also raise the possibility that human syndromes of idiopathic precocious and delayed puberty of central origin may have a previously unappreciated epigenetic component.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** GEO: GSE38505.

*Note:* Supplementary information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

A. Lomniczi and S.R.O. designed the project and wrote the paper. A. Lomniczi was involved in all aspects of the study. S.R.O. coordinated the project and performed the intrahypothalamic injections of viruses, including the imaging studies. A. Loche and J.M.C. ran global methylation arrays, ChIP and targeted methylation assays. O.K.R. and M.B. performed the single cell PCR experiments. G.K. measured mRNA by qPCR. J.G.K. determined the number of kisspeptin neurons in the ARC. H.W. designed the Perl script for the detection of HOTAIR consensus motifs and analyzed the data from DNA methylation arrays. G.P.P. advised us on methylation assays.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Animals. Rats. The use of rats (Sprague Dawley, Charles River Laboratories) was approved by the Oregon National Primate Research Center (ONPRC) Animal Care and Use Committee in accordance with the National Institutes of Health (NIH) guidelines for the use of animals in research. The rats were housed in a room with a controlled photoperiod (14/10 h light/dark cycle) and temperature (23–25 °C) and were allowed ad libitum access to tap water and pelleted rat chow.

To inhibit DNA methylation we treated rats with 5-azacytidine (Aza; 2 mg kg⁻¹ d⁻¹, i.p.), starting on postnatal day (P) 22. Control animals received the vehicle (saline solution, 2 ml kg⁻¹). Some animals were treated for 3 weeks to assess the time of puberty and estrous cyclicity. In all other studies, the animals were treated with Aza from P22 to P28.

To assess the estradiol response of the ovary to gonadotropins, immature control and Aza-treated rats were administered pregnant mare serum gonadotropin (PMSG; single s.c. injection, 8 IU per rat) at P26, and trunk blood was collected 2 d later for measurement of serum estradiol. The pituitary LH response to GnRH was assessed by injecting the decapetide (Sigma Aldrich, St. Louis, MO; 6.5 µg kg⁻¹, i.p.) and measuring LH in blood samples drawn 15 and 60 min later.

To determine whether overexpressing EED in the arcuate nucleus (ARC) of the hypothalamus would affect kisspeptin production, pulsatile GnRH release, the timing of puberty, estrous cyclicity and fecundity, female rats received on P22 an intrahypothalamic injection of lentiviral particles targeting EED to the ARC (details below). Some animals were killed 2 weeks after vaginal opening, and the number of immunopositive kisspeptin cells and the content of kisspeptin per cell in the ARC were assessed by immunohistofluorescence. Other animals were injected with the viral particles on P22, exposed to a fertile male for 5 d after all control (LV-GFP)-injected rats had undergone three complete estrous cycles, and their ability to deliver a litter evaluated. In a third experiment, the animals received the viral construct on P22, and pulsatile GnRH release from hypothalamic fragments containing the median eminence (ME)-ARC region was examined in vitro on P28.

Animals were randomly assigned to each treatment group. Mice. Female mice expressing enhanced green fluorescent protein (GFP) under the control of the 5′ flanking region of the mouse Kiss1 gene were bred and housed at the Oregon Heath and Sciences University (OHSU). All procedures performed using these animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the OHSU Animal Care and Use Committee.

Ovariectomy. Ovariectomy (OVX) of rats was performed at P24 under isoflurane anesthesia, and trunk blood was collected 96 h later for serum LH measurement. Ovariectomy of mice was similarly performed. All animals were given carprofen as an analgesic (4 mg kg⁻¹) before surgery.

Evaluation of sexual maturation and adult reproductive function. Rats were killed at four different peripubertal stages defined according to previously established criteria: EJ: early juvenile (21 d of age), LJ: late juvenile (28 d of age), EP: early proestrus (30–35 d of age) and LP: late proestrus (32–37 d of age). Animals in LJ exhibit a diurnal change in pulsatile plasma LH levels, with the LH pulses becoming more pronounced in the afternoon. This change is considered to be the first neuroendocrine manifestation of puberty. The first ovulation occurs the day after LP.

Once vaginal opening occurred, vaginal lavages were performed daily to identify the occurrence of the first estrus, which in rodents is manifested by a predominance of cornified cells. The age at first ovulation was considered to have occurred only when the cornified cells were followed by at least 2 d of lavages containing mostly leukocytes.

Trunk blood was collected on P28 for measurement of circulating LH, prolactin and corticosterone. At both P28 and P44, the ovaries were fixed in Kahle's fixative, embedded in paraffin, serially sectioned at 6 µm, stained with Weigert's iron hematoxylin and counterstained with picric acid–methyl blue for morphological evaluation.

Groups of animals receiving intrahypothalamic injections of lentiviral particles were killed at either P44 or after verifying their ability (or lack thereof) to deliver a litter when exposed to a fertile male, and their brains were perfusion-fixed for immunohistochemical verification of the site of injection (see below).

Incubation of ME and ARC-ME explants and measurement of GnRH release. Median eminence explants from rats treated for 7 d with either Aza or vehicle were incubated in vitro for 1 h under basal conditions and then for 1 h in the presence of 100 nM kisspeptin 110–119 (Phoenix Pharmaceuticals, Burlingame, CA). After another 1-h period of basal release, the tissues were stimulated for 1 h with 500 nM kisspeptin. GnRH released to the incubation medium was determined by RIA.

To estimate changes in pulsatile GnRH release from the ME-ARC of rats in which EED was overexpressed in the ARC via stereotaxic delivery of an EED-expressing LV construct, we used P28 rats that had received the LV construct on P22. The ME-ARC from animals receiving either LV-EED or LV-GFP was placed in Krebs-Ringer bicarbonate buffer and incubated for 4 h, removing the medium every 7.5 min for GnRH measurement. GnRH pulses were analyzed as described.

Measurement of GnRH, serum LH, estradiol, PRL and corticosterone levels. Assays for serum LH, prolactin (PRL), estradiol, corticosterone and gonadotropin-releasing hormone (GnRH) were performed by the Endocrine Technology and Support Lab, Oregon National Primate Research Center (Beaverton, OR).

RNA and DNA extraction. Total RNA and DNA were extracted using the AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA), according to the manufacturer's protocol.

Reverse transcription-PCR. Total RNA (500 ng) was transcribed into cDNA in a volume of 20 µl using 4 U Omniscript reverse transcriptase (Qiagen), and PCRs were performed using 1 µl of each reverse transcription reaction and SybrGreen ER (Invitrogen, Carlsbad, CA) in a volume of 10 µl. Ribosomal 18S RNA was measured in each sample as a housekeeping gene. Accession numbers of the genes amplified and primers sequences are shown in Supplementary Table 1.

Global DNA methylation. Global DNA methylation was determined in individual hypothalami using the Methylation Global DNA Methylation Quantification Kit (Epigentek, Brooklyn, NY) according to the manufacturer's protocol.

Methylation enrichment and quantification of DNA methylation. Methylated DNA was enriched using the EpiQuik Methylated DNA Immunoprecipitation Kit (Epigentek), following the procedure recommended by the manufacturer. For methylation array analysis, IP and input samples were amplified using the GenomePlex Complete Whole Genome Amplification (WGA2) Kit (Sigma) also following the manufacturer's instructions.

DNA methylation arrays. Immunoprecipitated and input samples were labeled with Cy5 and Cy3 respectively and hybridized to a Rat DNA Methylation 3x720K CpG Island Plus ReSeq Promoter Array (NimbleGen-Roche, Indianapolis, IN). The array procedure, scanning and data extraction were performed by the Center for Genome Research & Biocomputing at the Oregon State University. Methylation profiles were viewed using the NimbleGen SignalMap data browser.

Targeted DNA methylation-qPCR analysis. Validation of the DNA methylation arrays results was performed using a SybrGreen ER (Invitrogen) Real Time PCR analysis of target promoters using a QuantStudio 12K Flex Real Time PCR machine (Life Technologies, Carlsbad, CA). Accession codes of the genes whose promoter regions were amplified and primers sequences are shown in Supplementary Table 1.

Bioinformatics analysis of DNA arrays. After initial exploratory analysis of the peak signal data, we chose to use a rank-based approach to compare relative methylation between arrays and time points, as variation in absolute signal intensity between both time points and arrays within the time points was apparent. At least part of this variation may be attributed to the small number of biological replicates per time point (n = 3) used for the arrays. Probe regions were overlapped with the gene annotation regions given by the manufacturer, using the GenomicRanges Bioconductor package (http://www.bioconductor.org/) in R 2.15 (http://cran.r-project.org/). Although we first analyzed together both tracts provided by the array output—that is, one depicting methylation in CpG islands and the other depicting methylation of promoter regions—we also performed a
second analysis of promoter regions only, using 3 kb up- and downstream from the annotated TSS. For each annotation/promoter region, we took the mean ranking of all signal intensities overlapping that region as a summary of overall methylation of that feature. For each of the three time points (EI, LJ, and LP), we then determined the median summarized rank across the three arrays in each time point for each annotated region and compared the change in signal intensity rank between each pair of time points. From each comparison, we took the top 3,000 annotated regions with both largest positive and negative change in methylation rank each; each set of 3,000 genes was carried forward for functional analysis. Additionally, to determine whether any individual probe regions were hypermethylated in all three biological replicates of each time point, we used an empirical per-array cutoff of the 90th percentile of the distribution of all nonzero methylation signals among the probes on an array.

Using this gene set to search for transcriptional repressors we performed a functional analysis using the DAVID software (http://david.abcc.ncifcrf.gov/), version 6.7. In both the overall and promoter-specific analyses, we observed that genes with a pattern of changing methylation at either the LJ or the LP phases of puberty were functionally enriched for a cluster of chromatin/histone modification terms. Several of these genes were either members of the PcG silencing complex (Cbx7, Cbx8, Phc3, Rof2 and Ty1) or encoded proteins that interact with PcG proteins (Rybq, Cskx2b, Kdmz2b). All of them, with the exception of Rof2, exhibited a general pattern of increased methylation at puberty (LJ–LP). Although the combined analysis of promoters and CpG islands did not show a change in methylation for Cbx8 (Supplementary Fig. 2), analysis of only the promoter revealed increased methylation of this region at the LJ phase of puberty (Supplementary Fig. 2, inset).

Chromatin immunoprecipitation (ChIP) assays. These assays were performed using hypomethylated chromatin extracts, the antibodies described in Supplementary Table 2, and a procedure we previously described52.

PCR detection of chromatin-immunoprecipitated DNA. Genomic regions of interest were amplified by PCR. Accession codes of the genes analyzed, as well as the chromosomal position of the 5′-flanking region amplified, using the position of the transcription start site (TSS) as the reference point, are shown in Supplementary Table 1. The primer sequences (Eurofins MWG Operon, Huntsville, AL) used to detect the DNA fragment of interest in the immuno-precipitated DNA are also shown in Supplementary Table 1. PCR reactions were performed using 1 µl of each immunoprecipitated and input samples and SyberGreen ER (Invitrogen, Carlsbad, CA) in a volume of 10 µl.

Detection of Polycistron gene expression in GFP-expressing kisspeptin neurons by single-cell RT-PCR. To better visualize kisspeptin neurons of the ARC, we used 1-week ovariectomized adult mice. Single cells were harvested according to established procedures53 with minor modifications. In addition, aCSF harvested in the vicinity of the dispersed cells was used as a negative control. Cells and tissue RNA used as additional negative controls were processed as described above, but without reverse transcriptase (−RT).

Single cell RT-PCR was performed using 2 to 3 µl of cDNA template from each RT reaction in a 30 µl PCR mix. Fifty cycles of amplification were performed using a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA) according to established protocols53. PCR products were visualized with ethidium bromide on a 2% agarose gel.

Primer pairs designed to cross introns were selected using the Clone Manager software (Sci Ed Software, Cary, NC). The PCR products were sequenced to confirm the identity of the product. The primer sequences used are shown in Supplementary Table 1.

Fluorescence in situ hybridization. The brains from three immature P28 female rats were fixed by intracardiac perfusion of 4% paraformaldehyde borate buffer, pH 9.5, as previously reported41. The sections (25 μm) were cut on a frozen sliding microtome, mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), dehydrated under vacuum overnight, and then frozen at −80 °C until processing for hybridization. We employed a double fluorescence in situ hybridization procedure using a digoxigenin-11-UTP (DIG)-labeled Kiss1 cRNA probe transcribed from a rat Kiss1 cDNA, and fluorescein-12-UTP (FITC)-labeled probes recognizing either Eed or Cbx7 mRNA. Both labeling reactions were performed in a 10 µl volume. Eed mRNA encodes a PRC2 protein of the PcG silencing complex and Cbx7 mRNA a protein of the PRC1 subcomplex. The Eed cRNA probe (486 nucleotides (nt) in length) is complementary to nt 424–919 in rat Eed mRNA (NM_001106278.2) and the Cbx7 cRNA (474 bp) is complementary to nt 189–663 in rat Cbx7 mRNA (NM_199117). Control sections were incubated with sense probes transcribed from the same plasmid but were linearized on the 3′ end to transcribe the coding strand of the cDNA template.

Following treatment with proteinase K and acetic anhydride, the sections were hybridized overnight at 55 °C with Dig-Kiss1 cRNA in combination with either FITC-Eed or FITC-Cbx7 cRNA. The next day, the slides were washed at high stringency (final wash: 0.1× sodium chloride–sodium citrate at 65 °C for 30 min). Thereafter, the sections were incubated with 0.3% H2O2 for 10 min to block endogenous peroxidases, followed by 30 min in 0.5% blocking reagent provided with the Renaissance TSA Plus DNP system (PerkinElmer, Boston, MA) used to enhance the FITC reaction. Following these blocking steps, the sections were incubated overnight at 4 °C simultaneously with antidigoxigenin–alkaline phosphatase (AP)-conjugated sheep antibodies (Roche, Indianapolis, IN) diluted 1:1,000 and a mouse monoclonal anti-FITC conjugated to a peroxidase-conjugated IgG fraction (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:4,000 in TNT buffer (0.1 M Tris–HCl, 0.15 M NaCl, 0.5% Triton X-100). The next day, the sections were washed in TNT buffer (three times, 10 min each) before a 30 min incubation at 25 °C with tyramide signal amplification (TSA)-PLUS-dinitrophenyl (DNP) reagent (PerkinElmer) diluted 1:50. Following three washes in TNT buffer (10 min each), the sections were incubated with rabbit anti-DNP–keyhole limpet hemocyanin (KLH)–Alexa 488 (Invitrogen/Molecular Probes, Eugene, OR) diluted 1:500 in TNT buffer for 2 h at 25 °C to develop the FITC reaction into green fluorescence. Thereafter, the sections were again washed in TNT buffer (3 times, 10 min each time) followed by one wash in TSB0.0 buffer (0.1 M Tris HCl, pH 8.0, 0.1 M NaCl, 10 mM MgCl2); the digoxigenin reaction was then converted to red fluorescence by incubating the sections with HNPP (2-hydroxy-3-naphtoic acid-2-phenylalanide phosphate)/Fast Red reagent (Roche) for 30 min at 25 °C. After three washes with PBS-10 mM EDTA, the sections were incubated with Hoechst 33258 (Invitrogen) at 0.1 µg/ml for 1 min, washed in PBS and coverslipped with aqueous mounting medium before fluorescence microscopy examination.

Probes for in situ hybridization. Rat Kiss1, Cbx7 and Eed cRNA probes used were prepared by in vitro transcription of cDNA templates generated by RT-PCR amplification of hypomethylated total RNA. All PCR fragments were cloned into the pGEM-T vector (Promega) and their identity was verified by sequencing. Primer sequences and regions recognized by the cRNA probes used are shown in Supplementary Table 1.

Immunohistofluorescence and confocal microscopy. These procedures were performed using goat polyclonal antibodies to GFP (Abcam, Cambridge, Massachusetts, USA; 1: 2,000 dilution), followed by Alexa 488 donkey anti–goat IgG (Invitrogen, 1:500). Kisspeptin neurons were simultaneously identified using rabbit polyclonal antibodies to kisspeptin (Ab9754 from Millipore, diluted 1:500). The digoxigenin reaction was then converted to red fluorescence by incubating the sections with HNPP (2-hydroxy-3-naphtoic acid-2-phenylalanide phosphate)/Fast Red reagent (Roche) for 30 min at 25 °C. After three washes with PBS-10 mM EDTA, the sections were incubated with Hoechst 33258 (Invitrogen) at 0.1 µg/ml for 1 min, washed in PBS and coverslipped with aqueous mounting medium before fluorescence microscopy examination.

Counting of kisspeptin neurons and estimation of kisspeptin content per neuron. To estimate the number of ARC kisspeptin neurons with detectable kisspeptin immunoreactivity after in vivo injections of lentiviral particles overexpressing EED, images (10× magnification) were taken of the ARC region containing GFP (green)- and kisspeptin (red)-positive cells. The cells in each image were counted using the cell counter plug-in of ImageJ by an investigator blind to the treatment groups. Numerical raw data were entered into Excel, grouped by treatment and analyzed using Student’s t-test. ImageJ was used to measure the mean fluorescence intensity of manually outlined kisspeptin-positive cells containing GFP or devoid of GFP immunoreactivity. This procedure was also performed by an investigator blind to the treatment groups. Each outline was then moved over neighboring unlabeled areas to obtain a measure of the mean intensity of the background. Following subtraction of the background values form each cell, the mean intensity values per group were calculated and the statistical significance of the differences was calculated using a two-tailed Student’s t-test.

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Identification of putative PcG target motif in the Kiss1 promoter. Using a combination of Transfac analysis (http://www.biobase-international.com/product/transcription-factor-binding-sites/) and literature search, we examined 3 kb upstream of the rat Kiss1 promoter for several motifs found to be present in PcG target genes, including the core motif for YY1 binding (CCAT), the GAF (GAGAG) and extended MPho (CNGCCATNDNND) motifs, the two BMI1 binding motifs (CCTTCC and GGNNNGNG), and the binding motif for HOTAIR, a long noncoding RNA, recently shown to serve as an anchor for PcG binding to gene promoters38 (Supplementary Fig. 5a). As position-weight matrices were not reported for the binding motifs other than HOTAIR, we scanned for occurrences of all other motifs in the promoter using a custom Perl script and the reported consensus sequence. In the case of HOTAIR, we reconstructed the motif reported in ref. 38 on the basis of sequences provided by the laboratory of H.W. Chang (Stanford University), using the MEME tool (http://meme.nbcr.net/meme/) as reported in the reference. Our motif was then loaded as a custom PWM into the TRANSFAC motif discovery tool. We scanned for HOTAIR binding sites using a minimization of total false positive/negative (MinSUM) criteria.

Functional promoter assays. These assays were performed using HEK-293T cells and a luciferase reporter construct containing the 5′ flanking region of the Kiss1 gene, in addition to EED (see below) and/or YY1 expression vectors. Transfection efficiency was normalized by cotransfecting the plasmid CMV-β-gal (Invitrogen) at 10 ng/ml and determining β-galactosidase activity using the Tropix Galacto Reaction (ABI).

Preparation of EED-expressing lentiviral particles and production of viruses. To generate lentiviral particles expressing the rat Eed gene (LV-EED), we used a 3rd generation vector plasmid in which the promoter sequences of the 5′ LTR were replaced by the cytomegalovirus (CMV) promoter, resulting in the formation of a heterologous U3 (htU3) promoter26 (Supplementary Fig. 7a). The coding region of rat Eed tagged with the human influenza hemagglutinin epitope (HA) for easy detection by western immunoblotting was cloned into the BamHI–EcoRV sites of the plasmid's multiple cloning site. This cDNA is followed by an internal ribosome entry site (IRES) and a cDNA encoding an enhanced green fluorescent protein (eGFP). To enhance transgene expression, we inserted by an internal ribosome entry site (IRES) and a cDNA encoding an enhanced green fluorescent protein (eGFP). To enhance transgene expression, we inserted the coding region of rat Eed intron A sequence between the CMV promoter and the eGFP coding region of rat Eed intron A sequence between the CMV promoter and the eGFP (Supplementary Fig. 7a)36. Infective lentiviral particles were produced as previously described36.

Western blots. To verify the ability of the LV-EED construct to produce EED, we transfected COS7 cells with the construct using Lipofectamine 2000 (Invitrogen) and lysed the cells 48 h later with 500 µl of freshly prepared RIPA buffer. Antibodies to the HA epitope (Covance, MMS-101R) are described in Supplementary Table 2; they were used at a 1:2,000 dilution (overnight at 4 °C) followed by an species-specific anti-IgG–HRP antibody (1 h at 25 °C, 1:1,000; Invitrogen). The signal was developed by enhanced chemiluminescence using the Western lightning chemiluminescence substrate (Pierce).

Stereotaxic delivery of lentiviral particles. To deliver LV particles carrying the Eed transgene to the ARC, we used P26 early juvenile female rats and a procedure previously reported57.

Statistics. Quantitative data were analyzed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA). The data were first subjected to a normality test and an equal variance test. Data that passed these two tests were then analyzed by either ANOVA followed by the Student–Newman–Keuls multiple comparison test when comparing several groups or Student’s t-test to compare two groups. Because pulsatile GnRH release did not pass the equal variance test, these results were analyzed using the Mann–Whitney rank-sum test. When comparing percentages, groups were subjected to arc sine transformation before statistical analysis to convert them from a binomial to a normal distribution. The sample size was selected based on power analyses performed using the s.d. that we normally observe when measuring the parameters examined in this study and an n = 6 per group. These analyses provide at least 80% (type II error = 0.124) power to detect two effect sizes using either ANOVA or two-sided two-sample t-test with a significance level of 0.05.

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