Changes in Methylation Patterns of Kiss1 and Kiss1r Gene Promoters Across Puberty

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ABSTRACT: The initiation of mammalian puberty is underpinned by an increase in Kisspeptin (Kiss1) signaling via its receptor (Kiss1r/GPR54) on gonadotropin-releasing hormone (GnRH) neurons. Animals and humans with loss-of-function mutations in *Kiss1* or *Kiss1r* fail to go through puberty. The timing of puberty is dependent on environmental factors, and malleability in puberty timing suggests a mechanism that can translate environmental signals into patterns of *Kiss1/Kiss1r* gene expression. Epigenetics is a powerful mechanism that can control gene expression in an environment-dependent manner. We investigated whether epigenetic DNA methylation is associated with gene expression changes at puberty. We used bisulfite-PCR-pyrosequencing to define the methylation in the promoters of *Kiss1* and *Kiss1r* before and after puberty in female rats. Both *Kiss1* and *Kiss1r* showed highly significant puberty-specific differential promoter methylation patterns. By identifying key differentially methylated residues associated with puberty, these findings will be important for further studies investigating the control of gene expression across the pubertal transition.

KEYWORDS: puberty, methylation, kisspeptin, Kiss1r, neuroendocrine, reproduction

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Introduction

Puberty is the transitional period between childhood and adulthood in which reproductive capacity is achieved. Mammalian puberty onset is critically dependent on the activation of gonadotropin-releasing hormone (GnRH) neurons located within the rostral hypothalamus.[1] GnRH peptide stimulates the secretion of the gonadotrophins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), from the anterior pituitary gland; these, in turn, stimulate gonadal maturation, including steroid production.[1] Although the fertility-regulating circuitry develops during gestation and early neonatal life,[2–5] puberty occurs somewhat later, and mechanisms to explain this delayed activation of the GnRH neuronal network have been lacking.

Afferent inputs to the GnRH neuronal network that modulate puberty appear to change at or before puberty. There is an increase in stimulatory drive, in the form of glutamate and Kisspeptin1 (Kiss1) signaling,[6–9] and a decrease in inhibitory drive, in the form of opiate and GABA signaling.[10,11] Among the stimulators, Kiss1 is the most potent activator of GnRH firing and secretion described to date and has been shown to be absolutely required (along with its receptor Kiss1r) for puberty onset.[12–16] Interestingly, Kiss1 also appears to be involved in the very early development of GnRH neurons, where it controls their innervation of the median eminence.[5] Not surprisingly, loss-of-function mutations in *Kiss1* or *Kiss1r* result in failed puberty and infertility in both humans and animal models.[12,17,18]

Kiss1-expressing neurons that modulate puberty and reproduction reside in two main areas within the hypothalamus: the RP3V and the arcuate nucleus (ARC). Kiss1 neurons undergo a curious maturation, as many are generated

prenatally, but additional cells appear around the time of puberty in the RP3V.[19] Those in the RP3V do not initiate *Kiss1* expression until the late prepubertal period in-step with the rise in estrogen levels in the lead-up to the onset of GnRH neuron activation.[20,21] Indeed, estrogen is a strong stimulator of *Kiss1* expression in the RP3V both at puberty and as a positive feedback stimulator driving the LH surge during normal female reproduction.[22–24] The mechanism by which the initiation and upregulation of *Kiss1* gene expression in the RP3V is time-locked to the peripubertal period is unknown.

Recently, it was reported that global pharmacologic inhibition of DNA methylation prevented *Kiss1* upregulation in the mediobasal hypothalamus (MBH, the brain region containing the ARC Kiss1 neurons) and delayed puberty.[25] The underlying mechanism appears to be relief of a repressive chromatin state, mediated by Polycomb group silencers, which initiates upregulation of *Kiss1* expression in the MBH and onset of puberty.[25] However, Kiss1 expression in neurons of the RP3V have not been investigated. Because of the controversy surrounding the relative roles of the RP3V and ARC Kiss1 neurons in puberty, we investigated RP3V. We examined whether methylation of the *Kiss1* and *Kiss1r* promoters is altered along with puberty progression in the RP3V and POA, respectively, in female rats.

Kiss1 exerts its effects on GnRH neurons directly through its receptor, Kiss1r, which in the rostral hypothalamus is expressed primarily by GnRH neurons. GnRH neuron cell bodies are located in a rostro-caudal continuum from the medial septum to the anterior hypothalamic area.[1,26] Although not all reports are consistent, it appears that *Kiss1r* does not show a dramatic increase in expression in association with puberty.[26,27] Thus, *Kiss1r* represents a target that, although critical for puberty, is unlikely to undergo changes in methylation that alter its expression. However, it is possible that methylation changes at the time of puberty may act to ensure that *Kiss1r* expression is retained in the face of other major changes to the HPG axis.

Given the shared importance of *Kiss1r* and *Kiss1* expression for puberty, and the potential for these proteins to be differentially methylated in a manner that may reflect their distinct patterns of gene expression across puberty, the two were investigated. The goal of this study was to understand the changes in DNA methylation across the pubertal transition, particularly with respect to *Kiss1* and *Kiss1r*. For *Kiss1*, which is upregulated at and after puberty, we hypothesized that methylation changes would support this increased expression by either increasing binding by transcriptional activators or decreasing binding of transcriptional repressors. For *Kiss1r*, which is expressed both before and after puberty, we predicted that methylation changes would act to ensure that expression levels did not vary across puberty either by showing no changes in factor binding or by showing changes whose overall effects on transcription would not be different before

versus after puberty. We examined the methylation patterns for ~2 kb proximal promoter regions for *Kiss1* and *Kiss1r* genes before and after puberty in female rats. We defined methylation status and patterns for both genes before and after puberty and observed differential methylation at specific nucleotides in the promoters of these two genes before and after puberty and attempted to correlate these to both gene expression at the same ages and brain regions and potential transcription factor binding sites. The findings highlight specific loci within the promoters that may be differentially modified in physiological situations in which puberty timing is altered as well as provide a resource for further investigation of the epigenetic control of reproduction.

Results

Mapping of CpG dinucleotides in the promoters of Kiss1 and Kiss1r. Differential methylation of CpG dinucleotides in the promoter regions of mammalian genes have been associated with environmental factors and gene expression changes.[28–31] This study was conducted to examine whether *Kiss1* and *Kiss1r* promoter methylation changes across puberty in female rats and whether this is associated with gene expression changes. We initially mapped the distribution of CpG dinucleotides within the first approximately 2 kb 5' (upstream) of the transcriptional start site (Figs. 1E, F). Each promoter showed a unique number and pattern of CpG dinucleotides. *Kiss1* has 41 CpG/2.8 kb, and does not appear to have any well-defined CpG islands, although the density of CpGs does vary across the region (Fig. 1E). In contrast, *Kiss1r* has 71 CpG/2.2 kb, and two well-defined CpG islands—regions with >50% GC content, and a frequency of CpG dinucleotides greater than 60%,[31] one right at the transcription start site and another nearly 2 kb upstream (Fig. 1F).

Overall level of CpG promoter methylation differs between Kiss1 and Kiss1r and is not influenced by physiological state. Within the *Kiss1* and *Kiss1r* proximal promoters, the levels of overall methylation are strikingly different. The *Kiss1* promoter is mostly methylated irrespective of physiological state. Of the six regions surveyed in the *Kiss1* promoter, two had insufficient coverage for analysis and the remaining four showed that over 75% of all CpGs were methylated both before and after puberty (Fig. 2A). In contrast, the *Kiss1r* promoter was mostly unmethylated. Of the five regions surveyed in the *Kiss1r* promoter, one had insufficient coverage for analysis and of the remaining 4 most showed <20% CpG residues methylated (Fig. 2B). One exception was region 4, which showed nearly 60% methylated CpGs. This overall percentage of methylation in *Kiss1r*, like *Kiss1*, was irrespective of puberty.

In order to determine the relationship between promoter methylation and gene transcription, quantitative real-time PCR (qPCR) was used. Importantly, much of mRNA assessed by qPCR was derived from the same tissue as was
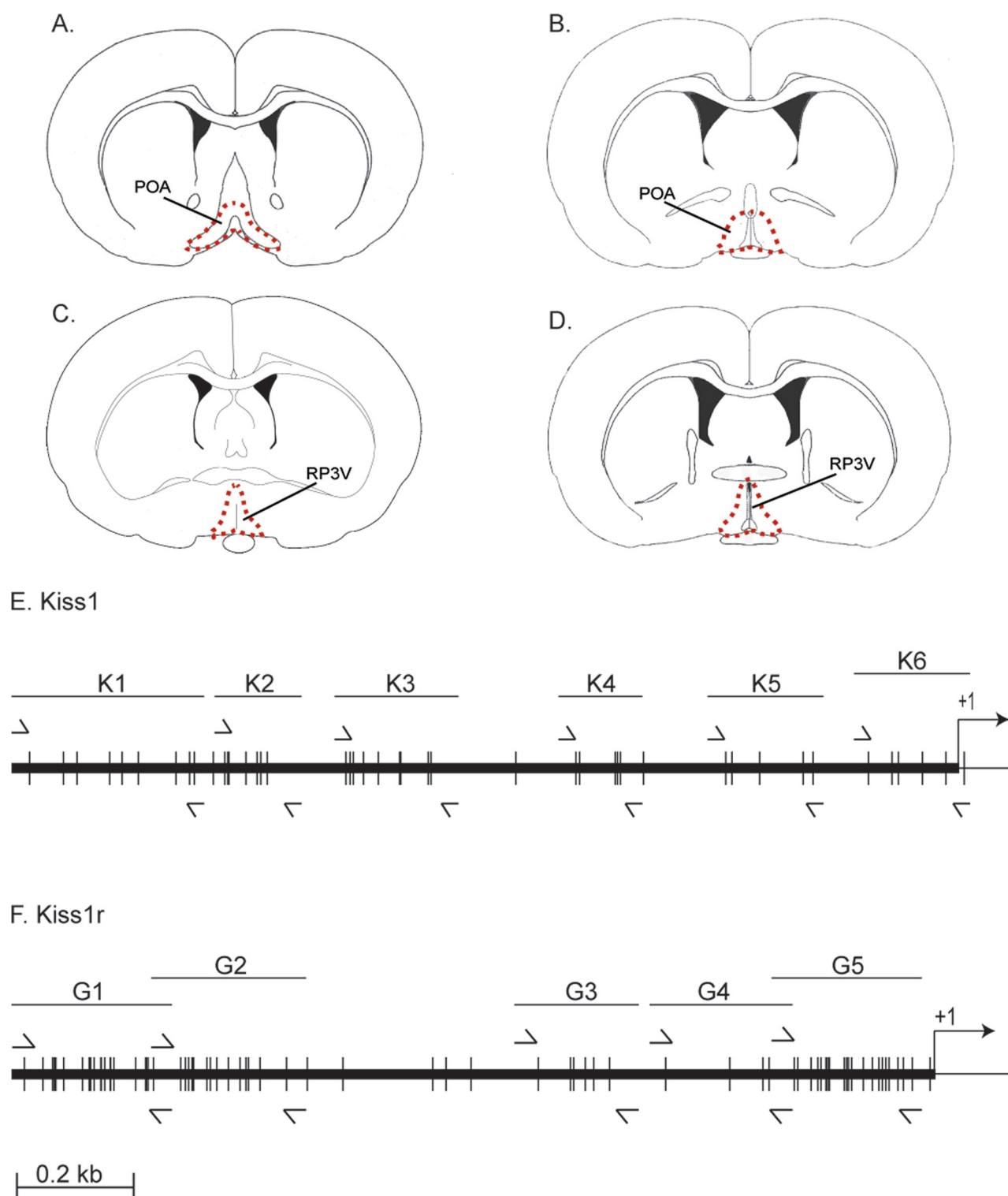
Figure 1. Details of hypothalamic microdissection and distribution of promoter CpG dinucleotides. **A–D.** show schematic diagrams of representative brain levels, modified from Allen Mouse Brain Atlas, Allen Institute for Brain Science (http://mouse.brain-map.org/experiment/thumbnails/100048576?image_type=atlas), in accordance with their usage policy (http://www.alleninstitute.org/Media/policies/citation_policy_content.html) from which tissue was taken. Dotted line demarcates region dissected and processed for DNA extraction and bisulfite sequencing. Rostral (**A**) and caudal (**B**) POA, containing GnRH neurons were pooled for Kiss1r analysis. Similarly, rostral (**C**) and caudal (**D**) regions of the RP3V were pooled for Kiss1 analysis. POA, preoptic area; RP3V, rostral periventricular region of the third ventricle. **E.** Schematic drawing of the *Kiss1* promoter showing positions of CpG dinucleotides (small vertical lines) and amplification primers (→ Forward, ← Reverse). +1 is the transcription start site and → indicates the direction of transcription. Note that in the ~2.8 kb 5′ to +1 that only 1 CpG is not covered by the primers. **F.** Schematic drawing of the *Kiss1r* promoter showing positions of CpG dinucleotides and amplification primers. Decorations are as in E. Note that in the ~2 kb 5′ to +1 that only 5 CpGs are not covered by the primers.

A. Kiss1
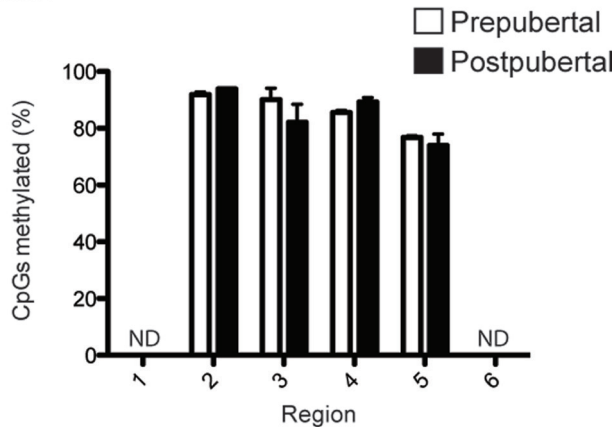
B. Kiss1r
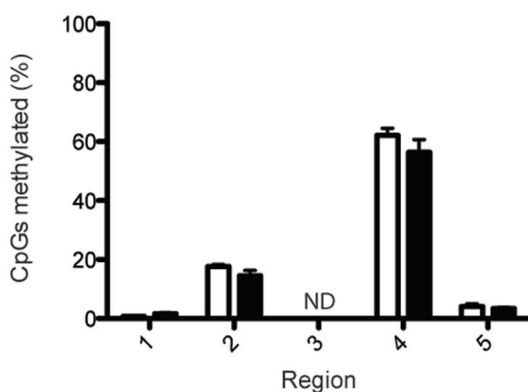
Figure 2. Details of methylation across entire promoter regions. **A.** Bar graph showing the percentage of CpG residues within each of the 6 regions across the *Kiss1* promoter. Note that the promoter as a whole is mostly methylated irrespective of physiological state. **B.** Bar graphs showing the percentage of CpG residues within each of the 5 regions spanning the *Kiss1r* promoter. Note that the promoter as a whole is mostly unmethylated irrespective of physiological state. ND, no data, indicating that insufficient sequencing coverage was obtained for these three regions. This mainly because of the high poly-T content that arises as a consequence of bisulfite conversion. Error bars indicate standard error of the mean.

used for methylation analysis (3/5 samples pre-pubertal and 2/5 post-pubertal for *Kiss1r*, and 1/5 samples pre-pubertal, and 2/5 post-pubertal for *Kiss1*). The remainder of samples were derived from different animals, but dissections used the same landmarks as for methylation analysis. This qPCR analysis showed no significant difference in *Kiss1r* expression between pre- and post-pubertal ages, which is consistent with previous observations (Fig. 3A, [26]). In contrast, *Kiss1* showed a significant increase in expression between pre- and post-puberty, again consistent with previous observations (Fig. 3B,).[32,33] When expression was then compared to overall percent methylation in *Kiss1r* (~23% methylated) and *Kiss1* (~80%), which remained static across puberty, there was no obvious association with gene expression level or change. Together these results suggest that gross,

or overall, levels of methylation across the 2 kb proximal promoter regions of *Kiss1* and *Kiss1r* do not correlate well to levels of expression for these two active genes at least within the RP3V and POA, respectively. The overall percentage of methylated residues across a region can be a useful predictor of transcriptional activity. However, methylation differences at individual nucleotide residues that may be bound by specific transcription factors, are also critical regulators of gene activity.

Kiss1 and Kiss1r promoters shows differential methylation across puberty. Despite a relatively static overall percent methylation over large regions, there were specific residues within each of the promoters that showed highly significant differences across puberty. The *Kiss1* promoter contained one residue (region 4, CpG 2) with significantly elevated methylation after puberty (Fig. 4A). None of the residues in any of the remaining CpG-containing regions showed significantly different methylation before versus after puberty (not shown). In contrast, the *Kiss1r* promoter possessed several residues with differential methylation across puberty (Figs. 4B–D). *Kiss1r* region 2 (Fig. 4B) is somewhat distant from the transcriptional start site, but may act as a distant enhancer. In contrast, regions 4 and 5 (Figs. 4C, D) are very close to the start of transcription, and may interact with proximal promoter-binding factors to control transcription. Thus, *Kiss1* and *Kiss1r* promoters showed differential methylation at specific residues that could affect transcriptional activity before versus after puberty.

In silico analysis of transcription factor binding sites and differential methylation. One approach to functionally dissect promoter regions with differential methylation is conduct in silico mapping of the positions at which transcriptional modulators may bind. Thus, in order to determine the functional significance of differential methylation patterns, in silico analysis of transcription factor binding sites was undertaken using the online Transcription Element Search System (TESS) resource (http://www.cbil.upenn.edu/cgi-bin/tess/tess).[34] Although the *Kiss1* promoter possessed only a single differentially methylated residue, this region appears likely to be bound by the transcriptional repressor Hes-1 (Fig. 5A).[35] This suggests that at puberty, the *Kiss1* promoter undergoes a switch from unmethylated Hes-1-binding, and thus transcriptionally inactive, to a methylated form that is not bound by Hes-1, and therefore is de-repressed and transcriptionally active. A similar conclusion was reached in a recent paper examining RP3V *Kiss1* promoter methylation in sexual dimorphism in mice.[36]

The *Kiss1r* promoter showed a number of differentially methylated sites that could be bound by transcription factors (Fig. 5B). Within region 2 and overlapping with CpG 12, there is a predicted binding site for GC-binding factor (GCF), a transcriptional repressor.[37] Within region 4 and overlapping with CpG 2, which loses methylation across puberty, there appear to be overlapping binding sites
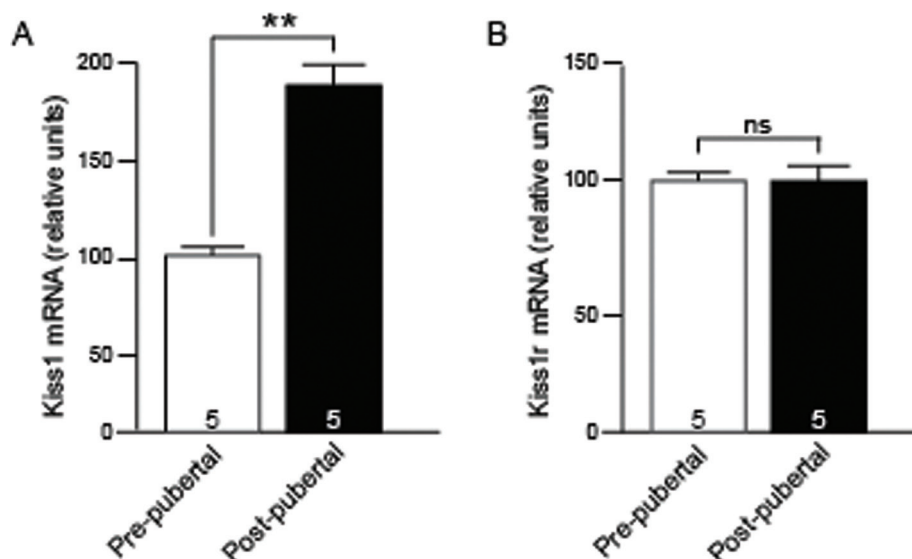
Figure 3. *Kiss1* and *Kiss1r* mRNA expression before and after puberty. Bar graphs showing the fold-change, relative to pre-puberty levels, in gene expression for *Kiss1* (**A**) and *Kiss1r* (**B**) before (white bars) and after (black bars) puberty. Numbers at base of bars indicate number of brains assayed. *, $P < 0.05$, Students t-test.

for NF-E and Sp1. Finally, region 5 contains CpG 9, which becomes increasingly methylated across puberty. This region is predicted to be bound by GCF and CCCTC binding factor (CTCF). CTCF, like GCF, is a well-described transcriptional repressor, and its binding has been shown to be regulated by differential methylation in cancer cells,[38] where hypermethylation leads to loss of CTCF binding and activation of transcription. Thus, both *Kiss1* and *Kiss1r* promoters show intriguing patterns of methylation changes across puberty, which may modulate the binding of specific transcription factors.

Discussion

This is the first comprehensive examination of methylation in the *Kiss1* and *Kiss1r* promoters from cells in the RP3V and POA, respectively, during puberty. Although recent studies have examined methylation in these promoters,[25,36,39] only very small regions of the promoters were examined. The data presented here show puberty-specific DNA methylation patterns spread across >2 kb in the promoters of both genes examined. In the case of *Kiss1r*, methylation differences did not appear to be associated with gross changes in gene expression, although they may indicate different modes of transcriptional control before and after puberty. In contrast, the *Kiss1* promoter exhibited differential DNA methylation in association with elevated expression coincident with puberty, but it is unclear whether the changes in methylation were causal. In support of a causal role, a recent report that examined the *Kiss1* promoter in the mouse RP3V also found increased methylation, suggesting a blockage of repressor binding to activate gene expression.[36] In mice and humans, specific regions within *Kiss1* or *Kiss1r* promoters that are

important for transcriptional activity have been identified.[40,41] Differential methylation or other epigenetic signatures in transcriptionally important promoter regions support that differential methylation is important for transcriptional control. Studies in which either the *Kiss1* or *Kiss1r* promoters were functionally dissected in rats, unfortunately, have not been performed. Moreover, the sequences of the promoter regions of rat *Kiss1* and *Kiss1r* differ considerably from those in mice, so a nucleotide-by-nucleotide methylation comparison is not possible. Thus, a functional comparison between methylation and transcriptional control awaits further study for these genes in the rat.

One feature of these data is that the changes are small. However, if a methylation change occurs even at a single nucleotide that is part of a transcription factor binding site, it could significantly influence whether the transcription factor binds. This is consistent with methylation changes in other genes, which may be small/subtle, but are nonetheless able to alter gene expression (see for example reference 30).
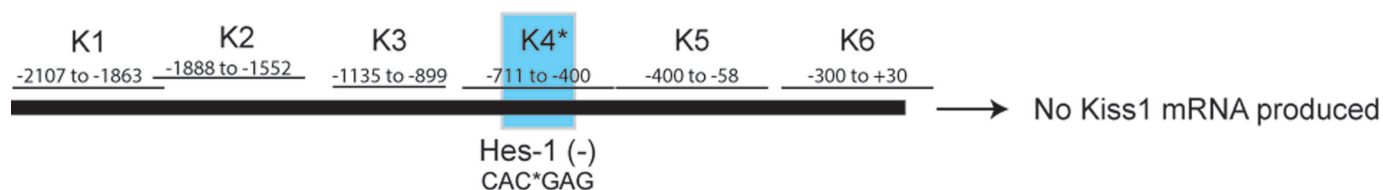
Interestingly, in terms of the overall amount of methylation at the >2 kb proximal promoter, irrespective of physiological state, the *Kiss1* promoter was mostly methylated compared to the *Kiss1r* promoter, which was mostly unmethylated. Overall levels of DNA methylation within promoters have been grossly correlated to gene activity.[31,42] Because methylated regions are more likely to form heterochromatin and thus less likely to be accessible to transcriptional machinery, they tend to be thought of as less likely to be expressed. Currently, however, a wide variety of methylation patterns have been observed within active promoters,[43,44] consistent with the gene-specific patterns we observed in this study; thus, the dogma of more methylation equals less expression is
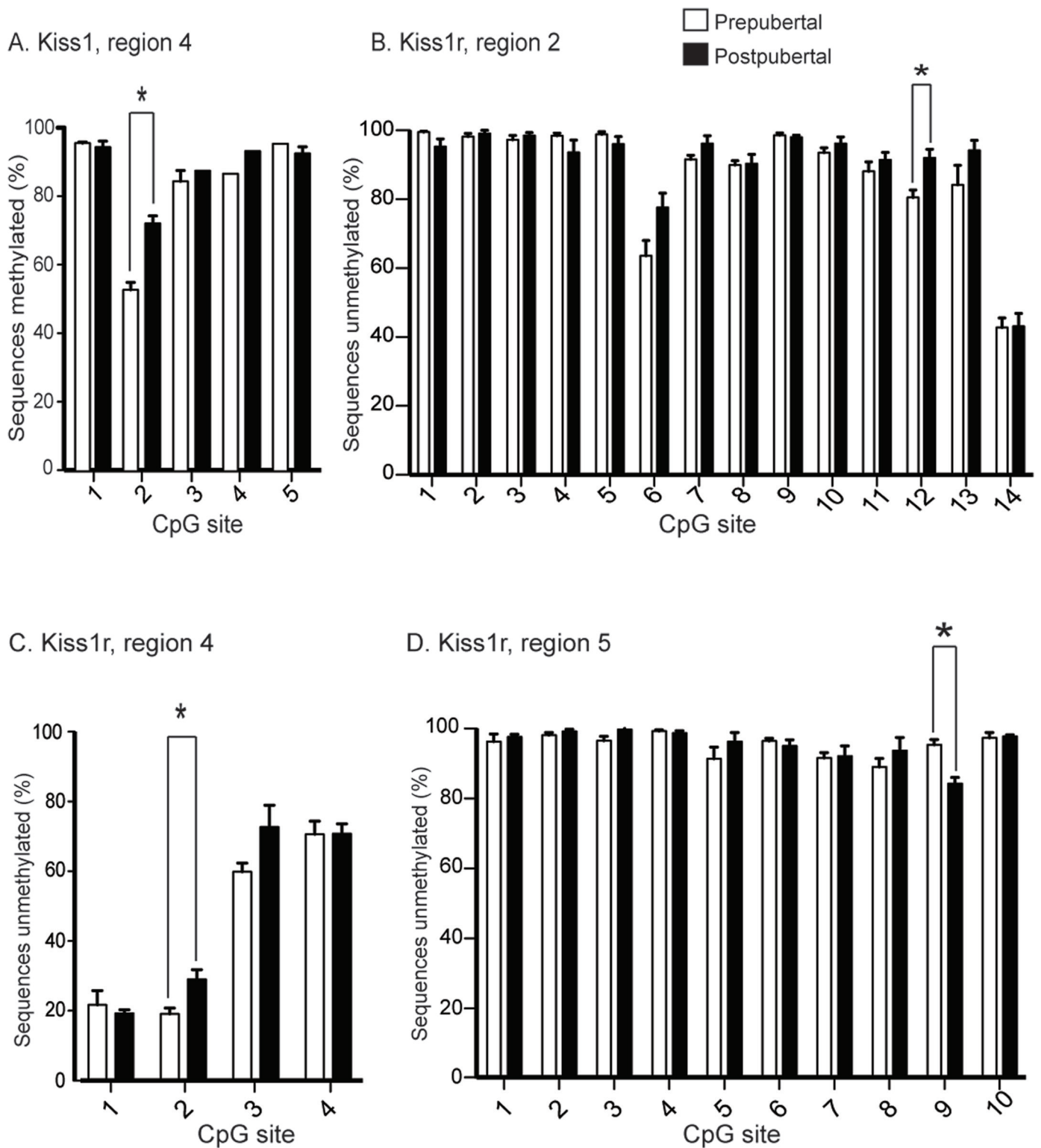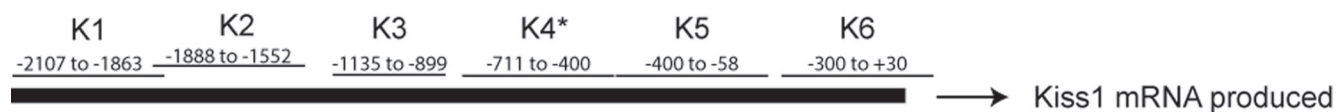
Figure 4. Differential methylation in the *Kiss1* and *Kiss1r* promoters across puberty. (**A**) Bar graph showing the percentage of CpGs that are methylated (y-axis) within region 4 of the *Kiss1* promoter before (white) and after (black) puberty. *$P < 0.05$, Student's t-test. (**B–D**) Bar graphs showing the percentage of CpGs that are unmethylated (y-axis) within regions 2 (**B**), 4 (**C**), and 5 (**D**) of the *Kiss1r* promoter before (white) and after (black) puberty. Note that regions 2 and 4 showed a decrease in methylation after (black bars) puberty compared with before (white bars; *, $P < 0.05$, Student's t-test), whereas region 5 showed and increase in methylation after puberty (*, $P < 0.05$, Student's t-test). Error bars indicate standard error of the mean. Due to the large number of reads sequenced per PCR reaction per animal, power analysis indicates >90% power in this experiment.
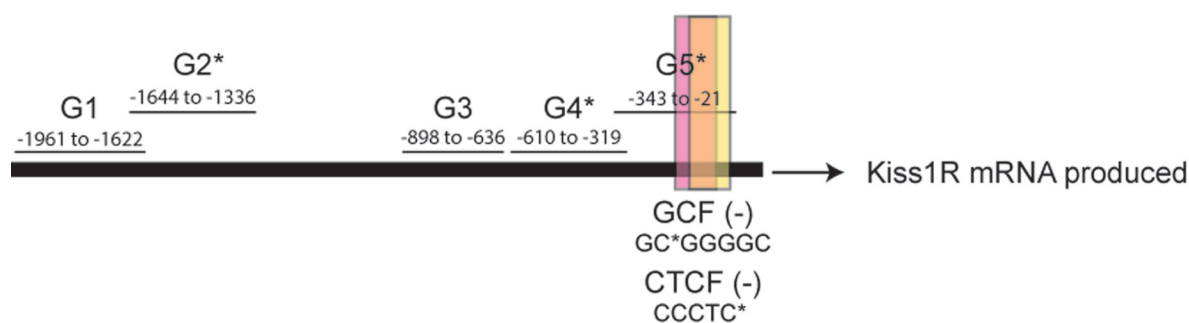
A. Kisspeptin1 (Kiss1)
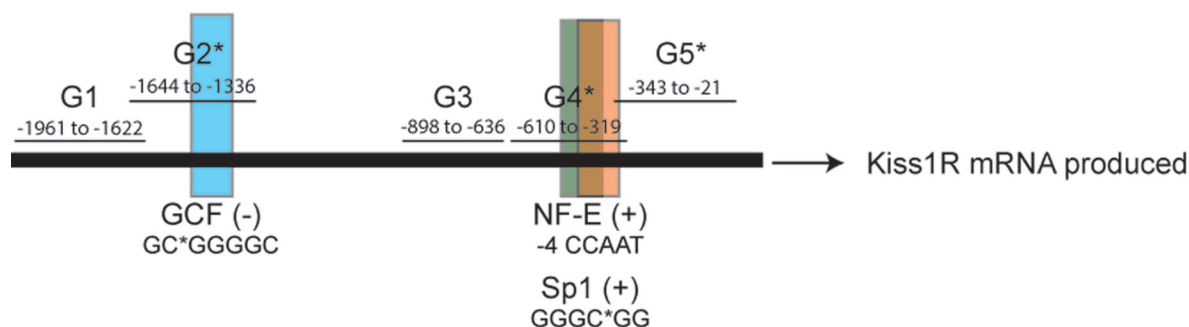
i.) Pre-puberty (transcriptionally inactive)

K1 -2107 to -1863
K2 -1888 to -1552
K3 -1135 to -899
K4* -711 to -400
K5 -400 to -58
K6 -300 to +30

→ No Kiss1 mRNA produced

Hes-1 (−)
CAC*GAG

ii.) Post-puberty (transcriptionally active)

K1 -2107 to -1863
K2 -1888 to -1552
K3 -1135 to -899
K4* -711 to -400
K5 -400 to -58
K6 -300 to +30

→ Kiss1 mRNA produced

B. Kisspeptin1 receptor (Kiss1r)

i.) Pre-puberty (transcriptionally active)

G2* -1644 to -1336
G1 -1961 to -1622
G3 -898 to -636
G4* -610 to -319
G5* -343 to -21

→ Kiss1R mRNA produced

GCF (−)
GC*GGGGC

CTCF (−)
CCCTC*

ii.) Post-puberty (transcriptionally active)

G2* -1644 to -1336
G1 -1961 to -1622
G3 -898 to -636
G4* -610 to -319
G5* -343 to -21

→ Kiss1R mRNA produced

GCF (−)
GC*GGGGC

NF-E (+)
-4 CCAAT

Sp1 (+)
GGGC*GG

Figure 5. Predicted binding sites for transcription factors relative to sites of differential methylation for *Kiss1* (**A**) and *Kiss1r* (**B**) promoters. Nucleotide numbers relative to the start of transcription are shown for each region examined. Each transcription factor predicted from the TESS analysis to bind to sites of differential methylation is shown. Sequences beneath transcription factor names denote core nucleotide binding sequence. Methylated residues within the core motif are shown with *. For NF-E, −4 indicates that the methylated C lies 4 nucleotides upstream of the 5'-most core sequence nucleotide. Factors are shown bound (present) when the promoter region is unmethylated and absent when the promoter regions is methylated. For each gene a the pre-puberty state (i) and the post-puberty state (ii) is shown. Dark horizontal bar represents promoter region sequenced, 2.8 kb for *Kiss1* and 2.2 kb for *Kiss1r*.

being revised. It is also possible that differences in methylation across the promoters of different genes may represent differences in the molecular manner in which gene expression is modulated; that is, a gene whose transcriptional regulatory regions are mostly methylated may require a fundamentally different mechanism for transcriptional activation by comparison with a gene whose promoter is mostly unmethylated. In the current situation, for example, *Kiss1* expression is under tight control to ensure that levels vary appropriately across the estrous cycle. In contrast, *Kiss1r* appears to be rather evenly expressed both before and after puberty, a relaxed level of control that may be mirrored in the mostly unmethylated promoter.

One technical caveat to the interpretation of these data is that they are derived from a mixed cell population, so it is possible that the level and pattern of cell-type specific methylation is underrepresented. Although global gene-specific patterns of methylation that correspond well to gene expression at puberty have been observed,[27] the ability to isolate genomic DNA from single GnRH and Kiss1 neurons both before and after puberty would be a strategy for further validation.

In silico analysis revealed a number of interesting possibilities regarding the binding of transcriptional modulators. For *Kiss1*, only a single factor, the transcriptional repressor Hes-1, was identified to potentially bind at the differentially methylated region. This is suggests a model in which puberty-specific methylation reduces Hes-1 binding in order to de-repress (ie, activate) the *Kiss1* promoter at the time of puberty, which is consistent with recent findings in mice.[36] Interestingly, E2 has been shown to repress *Hes-1* expression in breast cancer cells.[45] Should similar regulation occur in the RP3V Kiss1 neurons, rising E2 levels at puberty could cause Hes-1 to leave the *Kiss1* promoter, increasing levels of Kiss1 to increase GnRH release. However, the functional relationship among E2 levels, *Kiss1* promoter methylation, and transcription is unclear. Given the multiple functions of E2 and its assorted signaling pathways, it may both drive epigenetic changes and respond to them. Determining the exact role of E2 in the differential methylation patterns of *Kiss1* and *Kiss1r* associated with puberty, be it causal or casual, will required further study.

For *Kiss1r*, a number of differentially methylated sites were identified to which several transcriptional regulators putatively bind. NF-E was shown to interact with cyclic AMP response element-binding protein (CREB) binding protein (CREB-BP), a transcriptional activator involved in estrogen-responsiveness.[46] Previously, it was shown that E2-dependent methylation can modulate where CREB-BP binds the genome.[46] Another identified factor was Sp1, which has been previously reported to be a positive regulator of *Kiss1r* expression in mouse pituitary cells.[41] Finally, putative binding of the GCF and CTCF negative regulators was identified in a region of the *Kiss1r* promoter that undergoes

increased methylation across puberty. If this methylation blocks binding, GCF and CTCF may leave the promoter at the time of or after puberty. However, concomitant with this putative loss of binding, another site also bound by GCF loses methylation, and thus likely acquires GCF binding after puberty. The net result would be that negative regulation lost in one region of the promoter would be taken over by negative regulation at another region of the promoter, and thus no change in expression would occur. It is important to note that most of the *Kiss1r* promoter is unmethylated and it is actively expressed both before and after puberty. Thus, it is likely to be bound by a large number of transcription factors, making the role of any one factor difficult to determine from this analysis alone. Understanding whether these regions were differentially bound in vivo by the putative factors before versus after puberty would add compelling evidence for the significance of the differentially methylated sites in gene regulation at the time of puberty.

Importantly, this study did not address whether the methylation changes drive puberty directly or are related to activation of some other regulatory pathways (eg, via sex steroids), but rather cataloged how/whether methylation is different before and after puberty. One remaining key question that this study did not examine is whether gene expression changes that take place during the transition period are associated with specific methylation patterns that are different from those either before or after puberty. The regulation of all methylation changes—before, during, and after puberty—is likely to be quite complex and attributable to multiple pathways that act in concert to bring about the pubertal transition.

In conclusion, this study highlights key regions within both *Kiss1* and *Kiss1r* promoters that are differentially methylated within cells in the RP3V and POA, respectively, before and after puberty. The data presented here serve as a starting point for further investigation of the role of epigenetics in the transcriptional modulation of these genes by sex steroids and other environmental factors. It also offers the opportunity to examine the role of epigenetics in the differential regulation of *Kiss1* transcription between the RP3V and ARC at puberty and across the female reproductive cycle.

Materials and Methods

Animals. All experimental procedures were conducted with the approval of the University of Otago animal ethics committee. All experiments used female Sprague-Dawley rat brain tissue. Tissue was collected at two ages that spanned puberty: prepubertal (postnatal day 29 (PN29); n = 3) and postpubertal (PN80; n = 3), on diestrus. Animals in this study received common food and were reared and lived in identical environmental conditions (same room). In our colonies, female rats went through puberty, as judged by vaginal opening and first estrus, at an average age of 49 days.

Preparation of brain tissue and DNA and RNA extraction. Animals were killed by decapitation, and whole brains were removed, snap frozen on dry ice, and then stored at −80°C until use. Brains were cut into 300-μm sections using a cryostat and two hypothalamic regions were microdissected (the preoptic area (POA) and the rostral periventricular region of the third ventricle (RP3V)) for DNA and RNA extraction. The POA, which contains numerous GnRH neurons,[1] was located and then dissected from two consecutive sections using the anatomical landmarks set out in a stereotaxic coordinate atlas (Figs. 1A, B). The RP3V, encompassing both the anteroventral periventricular (AVPV) nucleus and the periventricular nucleus (PeN), and which contains numerous kisspeptin neurons,[20] was similarly dissected from two consecutive sections (Figs. 1C, D). Tissue was microdissected and then digested in 250 μL of lysis buffer following manufacturer's protocol (PureLink® genomic DNA kit, Invitrogen, Carlsbad, CA, USA). Concentrations of extracted DNA were measured using a nanospectrophotometer (NanoDrop® DN-1000, NanoDrop Products, Wilmington, DE, USA) and DNA was then stored at −20°C. Matched samples were also collected using the same dissection criteria (Figs. 1A–D) for preparation of mRNA. RNA was extracted using the Trizol reagent (Invitrogen, Australia) according to the manufacturer's protocol. RNA yield was determined on a Nanodrop spectrophotometer. Extracted RNA was then treated with DNase I (DNA-free™ kit, Ambion, Austin, TX, USA) according to the manufacturer's protocol to remove residual genomic DNA and reduce the potential for false-positive results in subsequent qPCR.

Quantitative reverse transcription-PCR. All qPCR was carried out with the LightCycler 480 Real Time PCR System (Roche, Basel, Switzerland), using white 96-well plates (Roche). SYBR Green chemistry was used for *Kiss1r* and Taqman chemistry was used for *Kiss1* PCRs. *Kiss1r* primers were designed using NCBI's primerBLAST function (http://www.ncbi.nlm.nih.gov/tools/primerblast), and *Kiss1* primers and Taqman probes were designed using Primer Express software (PE, Applied Biosystems, Foster City, CA, USA). Sequences of all primers and probes are shown in Table 1. For SYBR Green each individual reaction was run in triplicate for each RNA preparation and contained: 10 straliey app = "EN"mastermix (Applied Biosystems), 2 μm, forward and reverse mixed primer, 6 μL H$_2$O (Molecular grade, Invitrogen, Australia), and 2 μM cDNA (final cDNA concentration = 2 ng/mL). These reactions were run on the LightCycler using the following protocol: 95°C for 10 minutes (pre-incubation); 45 cycles of 95°C for 10 seconds, 60°C for 1 minute and 72°C for 30 seconds (amplification); 95°C for 1 second, 50°C for 1 minute (melting curve); 40°C for 30 seconds (cooling). Taqman reactions (20 μL) were prepared by adding 2 μL of template cDNA, in duplicate, together with LightCycler 480 Probes Master Mix (Roche) to 96-well LightCycler 480 Multiwell plates with adhesive covers (Roche). A reaction mix was prepared containing primers and probes at optimized concentrations of 500 nM and 200 nM, respectively, except the Kiss1 forward primer was used at a concentration of 300 nM. Samples were heated to 50°C for 2 minutes, then to 90°C for 10 minutes before 45 cycles of 95°C for 15 seconds and 60°C for 1 minute, as per the manufacturers instructions. All qPCR runs also included two types of negative controls: (i) no reverse transcriptase; (ii) no cDNA. All qPCR data were analyzed using the 2-ΔΔCt relative quantification method[47] to calculate fold-changes in gene expression relative to the internal standards (β2-microglobulin and β-actin) in the pre-pubertal and post-pubertal groups. Student's t-tests were performed to determine significance in gene expression change between the two groups.

Bisulfite conversion and PCR. To preserve the methylation status of the DNA, all extracted samples underwent bisulfite conversion as per manufacturer's instructions (Invitrogen™ MethylCode™ Bisulfite Conversion Kit). Bisulfite converted DNA was then stored at −20°C until use. Methylation-specific primer sets were designed using MethPrimer (http://www.urogene.org/methprimer/index1.html) to amplify portions of the upstream promoter regions of the *Kiss1* (2.7 kb, 6 primer sets) and *Kiss1r* genes (1.9 kb, 5 primer sets) that included the greatest number of CpG dinucleotides (for primer sequences see Table 2). *Kiss-1* primer sets were then used with RP3V samples and *Kiss1r* sets with POA samples, and subjected to PCR (PCR conditions for each primer set are detailed in Table 1) using Platinum *Taq* polymerase (Invitrogen). The PCR products were verified on a 1% agarose gel stained with ethidium bromide, and purified using a *Qiagen Min Elute PCR Purification Kit* following manufacturer's protocol (Qiagen, Hilden, Germany).

Table 1. qPCR primer sequences (5'–3').

GENE	FORWARD	REVERSE	PROBE
Kiss1	GCCGCTGGCAAAAGTGAA	GATTCCTTTTCCCAGGCATTAA	ATCCACAGGCCAGCAGTCCGGA
Kiss1r	GCTGGGAGACTTCATGTGCAA	AGCGGGAACACAGTTCACATACC	NA
beta-actin (Taqman)	AGGCCAACCGTGAAAAGATG	GCCTGGATGGCTACGTACATG	CCCAGATCATGTTTGAGACCTTCAAC
beta2-microglobulin	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC	NA
beta-actin	AGATGACCCAGATCATGTTTGAGA	ACCAGAGGCATACAGGGACAA	NA

Table 2. Methylation analysis primers (5'–3') and cycling parameters.

PRIMER NAME	SEQUENCE	ANNEALING TEMPERATURE (°C)	[MgCl$_2$] (mM)	PRIMER CONCENTRATION (μM)
Kiss1 region1, For	GAATTATTTGAAGGGAGTTTGGTAAG	58	1.5	0.5
Kiss1 region 1, Rev	CCTCTAACTCCCCTAAATACTCTAC			
Kiss1 region 2, For	GTTATGTGGGTTTAAGGGGTTAAAT	62	4	0.5
Kiss1 region 2, Rev	AATAATCCAAACTTCTACCTCTCATC			
Kiss1 region 3, For	TTTTATTATTTTTTTTGTTTAGAGA	54	3.5	0.8
Kiss1 region 3, Rev	ACAACTTATATAAATAAATCATTCC			
Kiss1 region 4, For	TAGTTGGGGGTTAGGTTAATTAATG	56	3	0.5
Kiss1 region 4, Rev	ACCCAAAACTTCTATTCAAAAACAA			
Kiss1, region 5, For	AGTTAAATTGATGAGGTTAATTTAGTT	60	4	0.5
Kiss1 region 5, Rev	TCAACTATTCTCTTAAAAACCTAAAAC			
Kiss1, region 6, For	TTTGTTTTGTAAATTTTTTATTTAGAG	58	3.5	0.5
Kiss1 region 6, Rev	CAACAACTACCAAAAAACCAAC			
Kiss1r region 1, For	TTTAGTTTAGGGAGGAGATTTTAGTT	58	0.75	0.5
Kiss1r region 1, Rev	AATTTTATCCTCAACCCTTCTAATC			
Kiss1r region 2, For	TTAGAAGGGTTGAGGATAAAATTTG	60	3	0.5
Kiss1r region 2, Rev	CTCCAATAATCCTCCTACCTCAAC			
Kiss1r region 3, For	TTTAGAGTTTTTAGATTTTTAATTTTT	58	3	0.5
Kiss1r region 3, Rev	CCTTATCAAACTACCAAAATACTATAC			
Kiss1r region 4, For	TTTTGAGATATATTTATTAGGGATGTTAGG	60	3	0.5
Kiss1r region 4, Rev	AAAAAATCATAACCCTTTCAAAAAC			
Kiss1r region 5, For	TTGAAAGGGTTATGATTTTTTTT	56	1.5	0.2
Kiss1r region 5, Rev	CCAAACAACCTTCACAAATTAC			

Barcoding and 454 sequencing. For each animal, all eleven purified PCR products, covering the *Kiss1* and *Kiss1r* promoters, were pooled according to Meyer et al.[48] The six pools were then barcoded using blunt-ended ligation.[48] For designing the barcodes, we used 8-bp long tags with a minimum substitution distance of 3, selected from the supplementary material in Meyer et al.[48] A pool of barcoded samples was then used to construct a sequencing library using the RLMID12 adaptors and sequenced on the 454 Life Sciences (Roche Diagnostics) GS Junior following the manufacturer's instructions.

Sequencing data analysis. 454 sequencing produced 36,384 sequencing reads in a single standard flowgram format (SFF). Obtained reads were initially examined using the Genome Sequencer FLX System Software version 2.3 and its SFF Tools commands (Roche Diagnostics). Only reads that contained complete individual-specific barcodes (with no mismatch) were extracted and separated into six individual SFF files for further analysis. The number of reads per individual ranged from 3826 to 6398. Individual SFF files were then imported into CLC Genomics Workbench 5.1 software (CLC Bio, Aarhus, Denmark), where reads were mapped to each of the eleven reference sequences. These reference sequences were modified using the assumption that all cytosine residues had been converted to thymines during bisulfite conversion. The CLC mapping parameters were set to the following: mismatch, insertion and deletion cost = 2, length fraction = 0.5, similarity fraction = 0.8. The mappings were then used for single-nucleotide polymorphism (SNP) detection. The criteria for SNP detection were set to minimum central and neighboring Phred quality score = 20, minimum coverage = 1, and minimum variant frequency = 0%. The list of detected SNPs was then narrowed to focus on previously determined CpG dinucleotides, and the percentage of C and T for each region was recorded. The reverse comparison was then completed, which mapped reads to references with the original cytosines still in place in order to identify CpG dinucleotides where 100% of the sequences had been converted to thymines, and thus would not be picked up in the initial mapping. The statistical differences in C/T percentages within each region of *Kiss1* and *Kiss1r* promoters between prepubertal and postpubertal groups were determined using unpaired, two-tailed t-test analysis (Prism, Graphpad Software, San Diego, CA, USA).

In silico transcription factor binding site analysis. Transcription Element Search System (TESS) (http://www.

cbil.upenn.edu/cgibin/tess/tess)[34] software was used to determine putative transcription factors that bind the promoter regions of *Kiss1* and *Kiss1r* genes. Genomic sequences corresponding to each amplicon plus an additional flanking ten base pairs were input into TESS for analysis using default parameter settings.

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Author Contributions

Conceived and designed the experiments: CLJ, AKW, JLV, NJG. Analyzed the data: CLJ, AKW, JLV, MZ, JLS. Wrote the first draft of the manuscript: CLJ, AKW. Contributed to the writing of the manuscript: AKW, MZ, JLV, JLS, NJG, CLJ. Agree with manuscript results and conclusions: AKW, MZ, JLV, JLS, NJG, CLJ. Jointly developed the structure and arguments for the paper: AKW, CLJ, NJG. Made critical revisions and approved final version: AKW, MZ, JLV, JLS, NJG, CLJ. All authors reviewed and approved of the final manuscript.

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