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—Original Article—

Retinoblastoma binding protein 7 is involved in *Kiss1* mRNA upregulation in rodents

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Abstract. Kisspeptin, encoded by *Kiss1*, is essential for reproduction in mammals. *Kiss1* expression is regulated by estrogen via histone acetylation in the *Kiss1* promotor region. Thus, elucidation of histone modification factor(s) involved in the regulation of *Kiss1* expression is required to gain further understanding of the mechanisms of its control. The RNA-seq analysis of isolated kisspeptin neurons, obtained from the arcuate nucleus (ARC) of female rats, revealed that *Rbbp7*, encoding retinoblastoma binding protein 7 (RBBP7), a member of histone modification and chromatin remodeling complexes, is highly expressed in the ARC kisspeptin neurons. Thus, the present study aimed to investigate whether RBBP7 is involved in *Kiss1* expression. Histological analysis using *in situ* hybridization (ISH) revealed that *Rbbp7* expression was located in several hypothalamic nuclei, including the ARC and the anteroventral periventricular nucleus (AVPV), where kisspeptin neurons are located. Double ISH for *Rbbp7* and *Kiss1* showed that a majority of kisspeptin neurons (more than 85%) expressed *Rbbp7* mRNA in both the ARC and the AVPV of female rats. Further, *Rbbp7* mRNA knockdown significantly decreased *in vitro* expression of *Kiss1* in a mouse immortalized kisspeptin neuronal cell line (mHypoA-55). Estrogen treatment significantly decreased and increased *Kiss1* mRNA levels in the ARC and AVPV of ovariectomized female rats, respectively, but failed to affect *Rbbp7* mRNA levels in both the nuclei. Taken together, these findings suggest that RBBP7 is involved in the upregulation of *Kiss1* expression in kisspeptin neurons of rodents in an estrogen-independent manner.

Key words: Kisspeptin neuron, *Kiss1*, Retinoblastoma binding protein 7

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Accumulating evidence suggests that kisspeptin-GPR54 (a kisspeptin receptor) signaling plays an essential role in regulation of reproductive functions via direct stimulation of gonadotropin-releasing hormone (GnRH) and consequent gonadotropin release in mammals, including rodents [1, 2], ruminants [3], primates [4], pigs [5], and musk shrews [6]. Mutations of *Kiss1* (encoding kisspeptin) or *Gpr54* cause hypogonadotropic hypogonadism in female rodents and humans [1, 2, 7–9]. Kisspeptin neurons are mainly located in two regions in the hypothalamus: one is located in the arcuate nucleus (ARC) in the mediobasal hypothalamus and the other is located in rostral hypothalamic regions, such as preoptic area (POA) in primates [10], ruminants [11, 12], and musk shrew [6], periventricular nucleus in pigs [5] or anteroventral periventricular nucleus (AVPV) in rodents [13, 14]. Kisspeptin neurons located in the ARC are suggested to be involved in follicular development and steroidogenesis via generation of pulsatile GnRH/gonadotropin secretion [15–19], while AVPV/POA kisspeptin neurons are suggested to be responsible for the ovulation via induction of GnRH/luteinizing hormone (LH) surge [14, 20–24].

During the few past years, emerging evidence has suggested that epigenetic mechanisms play a role in regulating *Kiss1* gene expression in the both ARC and AVPV [25–27]. We previously suggested that histone H3 acetylation of the *Kiss1* promoter region is involved in the upregulation of *Kiss1* mRNA expressions in both the nuclei in mice [27]: ARC *Kiss1* mRNA expression increases along with histone H3 acetylation of the *Kiss1* promoter region in the absence of estrogen, while estrogen increases AVPV *Kiss1* mRNA expression along with histone H3 acetylation of the *Kiss1* promoter region. Further, *in vitro* treatment with trichostatin A, an inhibitor of histone deacetylation, upregulated *Kiss1* mRNA expression in the mouse hypothalamic non-*Kiss1*-expressing cell line. Previous studies have also implied the epigenetic mechanisms underlying a pubertal increase in *Kiss1* mRNA expression in rats [28, 29]. Thus, polycomb repressive complex 2 (PRC2), a well-known transcriptional repressor complex, and sirtuin 1 (SIRT1), a histone deacetylase, are suggested to be involved in the prepubertal suppression of ARC *Kiss1* mRNA expression in rats [28, 29]. It is also reported that SIRT1 interacts with the PRC2 to decrease *Kiss1* promoter activity during the prepubertal period [29]. PRC2 reportedly catalyzes histone H3 trimethylation (also known as H3K27me3), a repressive histone modification [30]. Chromatin immunoprecipitation assay revealed a pubertal decrease in binding of embryonic ectoderm development (EED), a component of PRC2 [31], to the *Kiss1* promoter region [28]. The overexpression of

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EED causes suppression of *Kiss1* expression and subsequent GnRH secretion in rats [28]. For a further understanding of epigenetic mechanism underlying the regulation of *Kiss1* expression, roles of other histone modification-related proteins in *Kiss1* regulation should be investigated.

Retinoblastoma binding protein 7 (RBBP7), also named as retinoblastoma-associated protein 46 (RBAP46), has been reported to function as a histone chaperone in chromatin assembly and disassembly [32, 33]. RBBP7 is also known as a component of several histone modifications and chromatin remodeling complexes. It is well known that RBBP7 coupled with histone acetyltransferase 1 (HAT1) forms type B histone acetylation complex (HAT-B) that plays a key role in histone H4 acetylation in newly-synthesized histones in the cytoplasm [34]. Several reports indicate that RBBP7 forms two histone deacetylation complexes, NuRD and SIN3, that serve as the major transcription repressors in mammals [35]. Besides, RBBP7 also functions as a component of above-mentioned PRC2 [30]. Thus, we hypothesized that RBBP7 could be involved in the regulation of *Kiss1* expression in the hypothalamus.

The present study aimed to investigate the epigenetic mechanisms of *Kiss1* expression, focusing on the histone modification pathway. For this purpose, we first analyzed the expression of genes, products of which are related to the histone modification pathway using RNA-seq data of the isolated visualized kisspeptin neurons, obtained from the ARC of adult female *Kiss1*-tdTomato heterozygous rats, as previously described [36]. The analysis of the RNA-seq data revealed that substantial levels of *Rbbp7* mRNA were expressed in the ARC kisspeptin neurons. Thus, we performed histological analyses of the localization of *Rbbp7* mRNA in the hypothalamus and analyzed co-expression of *Rbbp7* and *Kiss1* transcripts in the ARC, as well as in the AVPV of female rats. Since we found that a majority of kisspeptin neurons co-expressed *Rbbp7* mRNA in both the ARC and the AVPV, we further determined the influence of RBBP7 on the regulation of *Kiss1* mRNA expression by analyzing the effect of siRNA-mediated knockdown of *Rbbp7* on *Kiss1* expression *in vitro* in a mouse immortalized ARC kisspeptin neuronal model (mHypoA-55 cells).

Materials and Methods

Animals and treatments

Female Wistar-Imamichi rats, 9–12 weeks of age (224–295 g body weight), were maintained under a controlled environment (14 h light and 10 h darkness; lights on at 0500 h; 22 ± 3°C) with free access to food (CE2; CLEA Japan, Tokyo, Japan) and water. Vaginal smears were checked daily to determine the estrous cyclicity and animals with at least two consecutive estrous cycles were selected for the experiment.

Five female rats were bilaterally ovariectomized (OVX) for 2 weeks to serve as OVX rats. Eleven female rats were OVX and immediately received subcutaneous Silastic tubing (1.57 mm inner diameter; 3.18 mm outer diameter; 25 mm in length; Dow Corning, Midland, MI, USA) filled with 17β-estradiol (E2; Sigma-Aldrich, St. Louis, MO, USA), dissolved in peanut oil (Sigma-Aldrich) at 20 μg/ml for 1 week to serve as OVX + low E2 rats (diestrous model). The low E2 treatment was previously confirmed to produce a negative feedback effect on LH pulses, but not to induce LH surges [37]. Five female rats were OVX and immediately received subcutaneous Silastic tubing filled with low E2 for 5 days, followed by Silastic tubing (1.57 mm inner diameter; 3.18 mm outer diameter; 28 mm in length) filled with E2, dissolved in peanut oil at 1000 μg/ml for 2 days to serve as the OVX + high E2 rats (proestrous model). The high E2 treatment was confirmed to induce daily LH surges (unpublished data). The OVX + low E2 rats were chosen so as to analyze the co-expression of the *Rbbp7* and *Kiss1* mRNA in both the ARC and the AVPV, because OVX + low E2 rats were confirmed to show *Kiss1* expression in both the nuclei [14]. All the surgical operations were performed under anesthesia of ketamine (27 mg/kg; Fujita, Tokyo, Japan) and xylazine (5.3 mg/kg; Bayer AG, Leverkusen, Germany) mixtures and isoflurane (1–3%, Pfizer, New York, NY, USA). All rats were injected with antibiotics (Meiji Seika Pharma, Tokyo, Japan) after surgery.

Care of the animals and experimental procedures were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University, Japan.

Preparation of female rat brain sections for in situ hybridization

The OVX + low E2 rats were deeply anesthetized with sodium pentobarbital (40 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) and perfused with 0.05 M phosphate-buffered saline, followed by 4% paraformaldehyde in 0.05 M phosphate buffer (PB). The brain was removed from the skull, postfixed with the same fixative, overnight at 4°C, and kept in 30% sucrose in 0.05 M PB for 3–4 days at 4°C under ribonuclease (RNase)-free conditions. Frozen coronal sections (50 μm in thickness) of the hypothalamus (from 0.60 mm anterior to 4.56 mm posterior to the bregma) according to a rat brain atlas [38] were obtained by using a cryostat, and subjected to single or double *in situ* hybridization (ISH).

Analysis of histone modification-related protein gene expression in ARC kisspeptin neurons obtained from Kiss1-tdTomato rats

Expression profiles of genes encoding histone modification-related proteins in ARC kisspeptin neurons were analyzed by using the RNA-seq data of isolated visualized kisspeptin neurons taken from the ARC of female *Kiss1*-tdTomato heterozygous rats, as described previously [36]. Gene symbols of histone modification-related proteins were obtained from the pathway portal of Rat Genome Database (histone modification pathway, http://rgd.mcw.edu/rgdweb/pathway/pathwayRecord.html?acc_id=PW:0001338). Since *Rbbp7* was highly expressed in ARC kisspeptin neurons, we further focused on the role of *Rbbp7* in the regulation of *Kiss1* expression.

Analysis of localization of Rbbp7 expression in the hypothalamus of female rats by single ISH for Rbbp7

Free-floating ISH for *Rbbp7* was performed by using the rat hypothalamic sections collected from OVX + low E2 rats (n = 3), as previously described [5, 39]. Briefly, *Rbbp7*-specific digoxigenin (DIG)-labeled cRNA probe (nucleotide 310-1424; GenBank accession no. NM_031816.1) was synthesized by using a DIG-labeling kit (Roche Diagnostics, Rotkreuz, Switzerland). The hypothalamic sec-

tions were hybridized overnight at 60°C with 1 µg/ml *Rbbp7*-specific DIG-labeled anti-sense cRNA probes. The specificity of the cRNA probe was confirmed by performing ISH by using anti-sense and sense probes. No signal was detected in the brain sections treated with a sense probe. After hybridization, the DIG-labeled probe was detected with alkaline phosphatase-conjugated anti-DIG antibody (1:500; Roche Diagnostics), and a chromogen solution (0.338 mg/ml 4-nitroblue tetrazolium chloride (NBT; Roche Diagnostics) and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP; Roche Diagnostics). The sections were mounted on gelatin-coated slides and examined under a light microscope (BX53; Olympus, Tokyo, Japan).

Analysis of co-localization of Rbbp7 and Kiss1 expression in the ARC and the AVPV of female rats by double ISH for Rbbp7 and Kiss1

Free-floating double ISH for *Rbbp7* and *Kiss1* was performed by using the hypothalamic sections including the AVPV (from the 0.48 mm anterior to 0.96 mm posterior to the bregma, every second section) and the ARC (from the 1.56 to 4.44 mm posterior to the bregma, every fourth section) gained from OVX + low E2 rats (n = 3), as previously described [36, 39]. Briefly, the *Rbbp7*-specific DIG-labeled cRNA probe and the *Kiss1*-specific fluorescein isothiocyanate (FITC)-labeled cRNA probe (position 33–348; GenBank accession no. NM_181692.1) were synthesized. The sections were hybridized overnight at 60°C with 1 µg/ml DIG-labeled *Rbbp7* anti-sense cRNA probes and 1 µg/ml FITC-labeled *Kiss1* anti-sense cRNA probes. After the hybridization, the FITC-labeled *Kiss1* probe on the sections was visualized by peroxidase (POD)-conjugated anti-FITC antibody (Roche Diagnostics) and TSA Plus Fluorescein System (1:100; PerkinElmer, Shelton, CT, USA). After inactivation of peroxidase in the sections with 0.1 N hydrochloric acid for 30 min, the DIG-labeled *Rbbp7* probe on the sections was visualized by POD-conjugated anti-DIG antibody (Roche Diagnostics), TSA Plus Biotin Kit (1:100; PerkinElmer) and Dylight 594-conjugated streptavidin (Thermo Fisher Scientific, Waltham, MA, USA). The sections were mounted to slides and the fluorescent images were examined under a fluorescence microscope with the ApoTome optical sectioning (Carl Zeiss, Oberkochen, Germany). The *Rbbp7*- and *Kiss1*-positive cells were counted bilaterally under the microscope, three times, by two persons and the average was calculated. No positive signal for *Rbbp7* or *Kiss1* mRNA was detected in the brain sections hybridized with the corresponding sense probes.

In vitro analysis of the role of Rbbp7 in regulating Kiss1 expression by Rbbp7 knockdown in mHypoA-55 cell line, a model for ARC kisspeptin neurons of mice

Immortalized mouse neuronal cells, mHypoA-55 cells (CVCL_D416; CELLutions Biosystems, Burlington, ON, Canada) were used as a model for ARC kisspeptin neurons [40] for *in vitro* analysis to investigate the role of *Rbbp7* in the regulation of *Kiss1* expression. The cells were cultured in phenol red-free Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) supplemented with 200 mM L-glutamine, 1% penicillin/streptomycin and 5% charcoal-stripped FBS (Biowest, Nuaillé, France) with or without E2 for 2 or 4 h. The E2 concentrations (10 or 100 nM) and incubation period (2 or 4 h) were chosen according to a previous study [40], in which Treen *et al.* showed that the 4 h treatment of 100 nM E2 decreased *Kiss1* expression in mHypoA-55 cells, as well as to our preliminary investigations that revealed decreasing tendency of *Kiss1* expression upon E2 treatment using the same cell lines.

The effect of *Rbbp7* knockdown on *in vitro Kiss1* expression in the mHypoA-55 cells was determined using Silencer Select Pre-Designed siRNA (10 nM), corresponding to mouse *Rbbp7* (siRNA ID: s110672 (#1); s110673 (#2), Thermo Fisher Scientific). The 2 sets of *Rbbp7*-siRNAs or negative control siRNA (NC siRNA) were transfected into the cells by Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific). Following this, 48 h after the transfection, the cells were treated with 100 nM E2 for 2 h, and the cells were collected for analyses for *Kiss1* and *Rbbp7* mRNA or RBBP7 protein levels. The E2 treatment was chosen due to the observed tendency of a decrease in *Kiss1* expression following 100 nM E2 treatment for 2 h.

Quantitative RT-PCR analyses for Rbbp7 and Kiss1 mRNA expression levels in the ARC and the AVPV regions of the rats and mHypoA-55 cells

To analyze *Rbbp7* and *Kiss1* mRNA levels in the ARC or AVPV regions of OVX, OVX + low E2, and OVX + high E2 rats (n = 5 in each group), animals were decapitated between 1300 h and 1500 h. The ARC-median eminence (ME) region was dissected from the brain with a microblade and the AVPV region was punched out with 18-gauge stainless-steel tubing as previously described [41]. *Rbbp7* and *Kiss1* mRNA levels were also analyzed in the mHypoA-55 cells described above.

DNA-free total RNA was purified from the ARC and AVPV tissues, as well as mHypoA-55 cells by using ISOGEN (Nippon Gene, Tokyo, Japan). For ARC or AVPV tissues, DNase I (Thermo Fisher Scientific) and a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) were used to synthesize full-length cDNAs. For mHypoA-55 cells, ReverTra Ace (TOYOBO, Osaka, Japan) was used to synthesize full-length cDNAs. The obtained cDNA was used as a template in PCR reactions using primers for *Rbbp7*, *Kiss1* and *Actb*. Forward and reverse primers for rat *Kiss1* were 5'-AGCTGCTGCTTCTCCTCTGT-3' and 5'-AGGCTTGCTCTCTGCATACC-3', respectively (GenBank accession no. NM_181692.1); for mouse *Kiss1*, 5'-TGCTGCTTCTCCTCTGT-3' and 5'-ACCGCGATTCCTTTTCC-3' (GenBank accession no. NM_178260.3); for rat and mouse *Rbbp7*, 5'-TCCCAATGATGATGCACAGT-3' and 5'-CTGGTTTTGCAGGGTGTTTT-3' (GenBank accession no. NM_031816.1 and NM_009031.3); for rat *Actb*, 5'-TGTCACCAACTGGGACGATA-3' and 5'-GGGGTGTTGAAGGTCTCAAA-3' (GenBank accession no. NM_031144.3); for mouse *Actb*, 5'-GGTGGGAATGGGTCAGAAGG-3' and 5'-GTACATGGCTGGGGTGTTGA-3' (GenBank accession no. NM_007393.5). Real-time PCR analysis was performed by using an ABI 7500 real-time system (Thermo Fisher Scientific) with THUNDERBIRD qPCR Mix (TOYOBO). The cycling protocol was as follows: pre-denature for 1 min at 95°C, 40 cycles amplification for 15 sec at 95°C and 1 min at 60°C. The specificity of the amplification products was confirmed by dissociation curve analysis (60 to 95°C) after 40-cycle amplification. A distinct single peak was considered as

proof that only a single DNA sequence was amplified. The expression levels of *Rbbp7* and *Kiss1* genes were obtained by normalization to the expression levels of *Actb*.

Verification of Rbbp7 knockdown experiment in mHypoA-55 cells by western blotting for RBBP7

The expression levels of RBBP7 and β-actin proteins in the mHypoA-55 cells after 48 h of siRNA transfection were confirmed by western blotting. The cells cultured with *Rbbp7*-siRNAs or NC siRNA were treated with 10% (v/v) trichloroacetic acid (Nakarai tesque, Kyoto, Japan) at 4°C for 30 min, and scraped off. These cells were then suspended in UTD buffer [9 M Urea (Sigma-Aldrich), 2% (v/v) Triton X-100 (Sigma-Aldrich), 1% (w/v) DL-Dithiothreitol (DTT; Sigma-Aldrich)] and 2% (w/v) lithium dodecyl sulfate (Sigma-Aldrich). The whole cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA), and transferred onto polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA). The membranes were incubated with rabbit anti-human RBBP7 polyclonal antibody (1:4,000 Abcam, Cambridge, UK) at room temperature for 2 h. The specificity of the antibody was confirmed by He *et al*. [42]. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:4,000 Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for 1 h. Chemiluminescence was visualized using an ECL Prime Western Blotting Detection Reagent (GE Healthcare), and recorded with a Light-Capture II (AE-6982; Atto, Tokyo, Japan). The membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) for re-probing with rabbit anti-human β-actin polyclonal antibody (1:10,000 Abcam). The expression of β-actin protein was detected by using the same method as stated above and its levels were used as an internal control. Band intensities were estimated by densitometry using Image J software v. 1.52k (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical differences in *Rbbp7* and *Kiss1* mRNA and RBBP7 protein levels in the mHypoA-55 cells, transfected with siRNAs and treated with E2 were determined by two-way ANOVA (siRNA transfection and E2 treatment as main effects) with Bonferroni post-hoc test. Statistical differences in *Rbbp7* and *Kiss1* mRNA levels in the ARC and the AVPV of rats or in the mHypoA-55 cells treated with three different doses of E2 were determined by one-way ANOVA with Bonferroni post-hoc test. $P < 0.05$ was considered to indicate a significant difference. All analyses were performed by using R Statistical Computing, release 3.5.1 (http://www.R-project.org).

Results

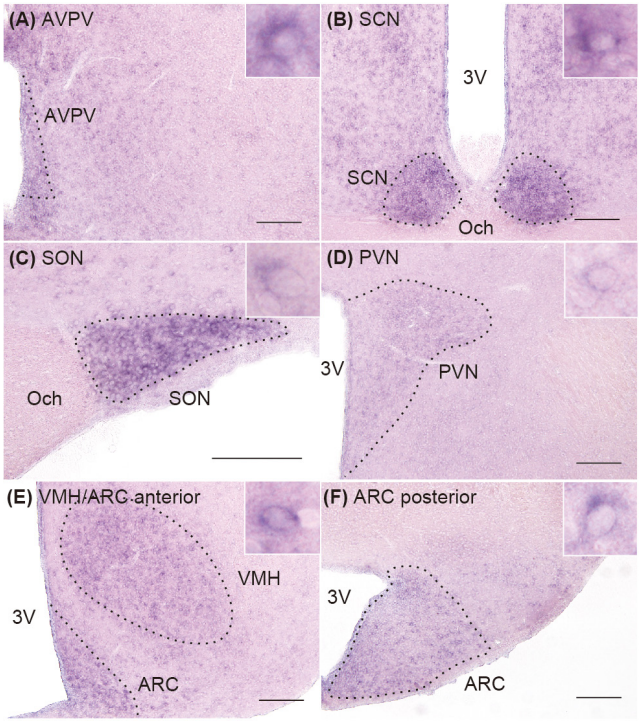
Examination of histone modification factor gene expression in the ARC kisspeptin neurons by analyzing the RNA-seq data obtained from isolated rat ARC kisspeptin neurons

The RNA-seq analysis showed that some major histone modification factor genes (Rat Genome Database, http://rgd.mcw.edu/rgdweb/pathway/pathwayRecord.html?acc_id=PW:0001338), such as *Rbbp7* (210 RPKM), *Hdac1* (encoding histone deacetylase 1, 63 RPKM), *Hdac2* (encoding histone deacetylase 2, 35 RPKM), *Ep300* (encoding E1A binding protein P300, 1.42 RPKM), and *Sirt1* (Sirtuin 1, 0 RPKM) were detected in ARC *Kiss1*-tdTomato cells. Among these genes, *Rbbp7* was expressed at high levels in the cells. Table 1 shows genes whose transcripts form histone modification complexes together with RBBP7, such as NuRD, SIN3, PRC2, and HAT-B complexes. ARC *Kiss1*-tdTomato cells expressed the component genes of NuRD and HAT-B complexes reported previously [35, 43]. On the other hand, few component genes of SIN3 and PRC2 complexes, such as *Sap30* and *Eed* [35, 44], were detected in the ARC *Kiss1*-tdTomato cells.

Table 1. Genes whose transcripts form histone modification complexes together with retinoblastoma binding protein 7 (RBBP7)

Gene symbols	Relative expression levels *	Histone modification complexes
Rbbp7	209.55	NuRD, SIN3, PRC2, HAT-B
Rbbp4	127.84	NuRD, SIN3, PRC2, HAT-B
Hdac1	62.70	NuRD, SIN3
Hdac2	34.67	NuRD, SIN3
Gatad2b (p66β)	23.26	NuRD
Chd4	17.15	NuRD
Mbd3	12.14	NuRD
Gatad2a (p66α)	9.94	NuRD
Chd3	6.73	NuRD
Mta1	0.56	NuRD
Mta2	0.06	NuRD
Sap18	150.35	SIN3
Sin3b	69.22	SIN3
Sin3a	4.18	SIN3
Sap30	0.00	SIN3
Suz12	3.10	PRC2
Ezh1	0.00	PRC2
Ezh2	0.00	PRC2
Eed	0.00	PRC2
Hat1	8.83	HAT-B

* Data are expressed as reads per kilobase per million mapped reads (RPKM). Symbols and names of gene or histone modification complexes are as follows: *Chd3*, Chromodomain helicase DNA binding protein 3; *Chd4*, Chromodomain helicase DNA binding protein 4; *Eed*, Embryonic ectoderm development; *Ezh1*, Enhancer of zeste 1 polycomb repressive complex 2 subunit; *Ezh2*, Enhancer of zeste 2 polycomb repressive complex 2 subunit; HAT-B, Type B histone acetyltransferases; *Hat1*, Histone acetyltransferase 1; *Hdac1*, Histone deacetylase 1; *Hdac2*, Histone deacetylase 2; *Mbd3*, Methyl-CpG binding domain protein 3; *Mta1*, Metastasis-associated proteins 1; *Mta2*, Metastasis-associated proteins 2; NuRD, Nucleosome remodeling and deacetylation; *Gatad2a* (*p66α*), GATA zinc finger domain containing 2A (Transcriptional repressor p66-alpha); *Gatad2b* (*p66β*), GATA zinc finger domain containing 2B (Transcriptional repressor p66-beta); PRC2, Polycomb repressive complex 2; *Rbbp4*, Retinoblastoma binding protein 4; *Rbbp7*, Retinoblastoma binding protein 7; *Sap18*, Sin3A associated protein 18; *Sap30*, Sin3A associated protein 30; SIN3, Sin3 histone deacetylase complex; *Sin3a*, SIN3 transcription regulator family member A; *Sin3b*, SIN3 transcription regulator family member B; *Suz12*, SUZ12 polycomb repressive complex 2 subunit.



Distribution of the Rbbp7 mRNA-expressing cells in the hypothalamus of female rats

Figure 1 shows the *Rbbp7* mRNA expression in the hypothalamus of a representative OVX + low E2 rat. A number of *Rbbp7*-positive cells were found in the AVPV (Fig. 1A), suprachiasmatic nucleus (SCN) (Fig. 1B), supraoptic nucleus (SON) (Fig. 1C), paraventricular nucleus (PVN) (Fig. 1D), anterior part of the ventromedial hypothalamic nucleus (VMH) and the ARC (Fig. 1E), and the posterior part of the ARC (Fig. 1F).

Co-expression of Rbbp7 in Kiss1-positive cells in the ARC and AVPV of female rats

Figures 2A and 2B show *Rbbp7*- and *Kiss1*-positive cells, visualized by a double ISH in the ARC and AVPV of representative OVX +

Fig. 1. Distribution of *Rbbp7*-positive cells in the hypothalamus of ovariectomized + low 17β-estradiol (OVX + low E2) rats. Representative photomicrographs showing cells expressing *Rbbp7* in the anteroventral periventricular nucleus (AVPV) (A), suprachiasmatic nucleus (SCN) (B), supraoptic nucleus (SON) (C), paraventricular nucleus (PVN) (D), anterior part of the ventromedial hypothalamic nucleus (VMH) and arcuate nucleus (ARC) (E), and posterior part of the ARC (F). The insets indicate *Rbbp7*-positive cells at a higher magnification. Scale bars: 200 μm. 3V, third cerebroventricle; Och, optic chiasm.

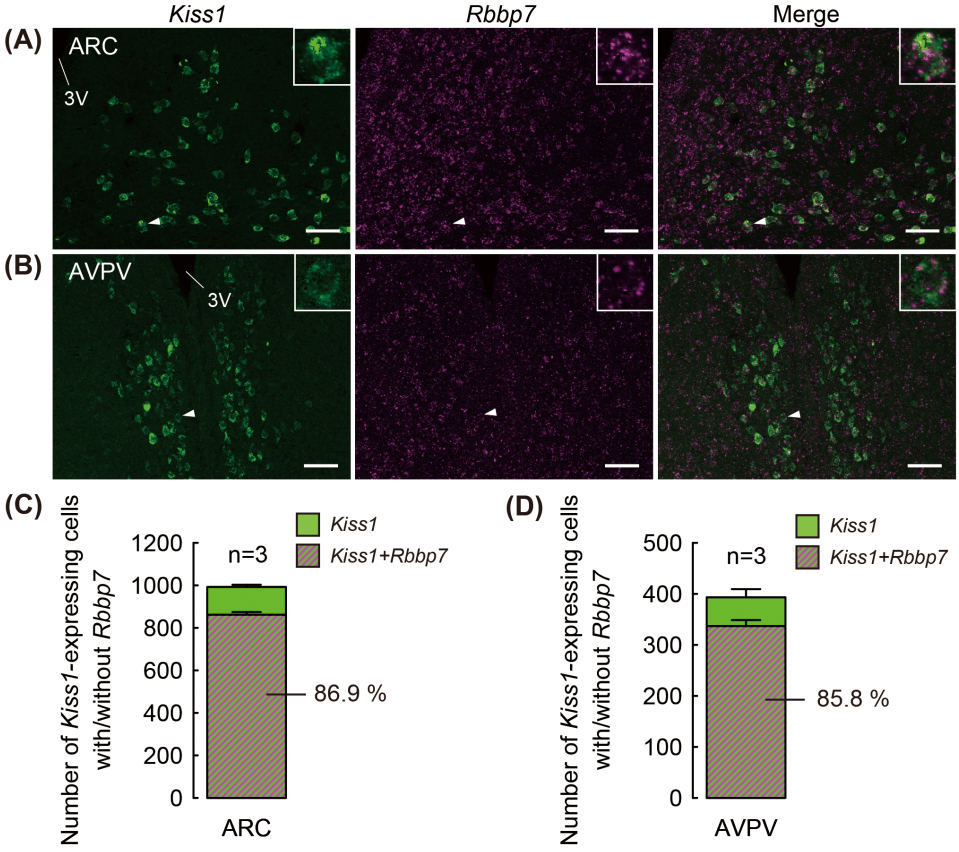


Fig. 2. Co-expression of *Kiss1* and *Rbbp7* in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) of ovariectomized + low 17β-estradiol (OVX + low E2) rats. Representative photomicrographs showing *Kiss1*-positive cells (green) and *Rbbp7*-positive signals (magenta) in the ARC (A) and AVPV (B). The insets indicate expression of *Rbbp7*-positive signals in the *Kiss1*-positive cells at a higher magnification (arrowheads). The numbers of *Kiss1*- and *Rbbp7*-positive cells out of the *Kiss1*-positive cells were quantified in the ARC (C) and AVPV (D). Data are the mean ± SEM (n = 3). Scale bars: 100 μm. 3V, third cerebroventricle.

low E2 rats. Cell-shaped *Kiss1*-positive signals were found in the both ARC and AVPV and dot-like *Rbbp7*-positive signals were widely found in the ARC and the AVPV and outside of the nuclei. Merged images exhibit that most of the *Kiss1*-positive cells showed co-expression of *Rbbp7*-positive signals in both, the ARC (Fig. 2A) and the AVPV (Fig. 2B). Quantitative analysis revealed that the majority of *Kiss1*-positive cells co-expressed *Rbbp7* in the ARC (86.9%, Fig. 2C) and the AVPV (85.8%, Fig. 2D) of the OVX + low E2 rats.

Suppression of Kiss1 expression by Rbbp7 silencing in the mHypoA-55 cell line

Both *Rbbp7*-targeting siRNAs #1 and #2 significantly reduced *Rbbp7* mRNA (Fig. 3A) and protein (Fig. 3B) expression, regardless of E2 treatment in the mHypoA-55 cells ($P < 0.05$, two-way ANOVA with Bonferroni post-hoc test). The knockdown of *Rbbp7* mRNA by the siRNA #1 or #2 significantly decreased the *Kiss1* mRNA levels in the cells, regardless of E2 treatment (Fig. 3C; $P < 0.05$, two-way ANOVA with Bonferroni post-hoc test).

Quantitative analysis of Rbbp7 and Kiss1 expression in the mHypoA-55 cells treated with/without E2 and in the ARC and the AVPV of OVX rats treated with/without diestrous (low) and proestrous (high) levels of E2

Figures 4 A–C show *Rbbp7* and *Kiss1* expression levels determined by quantitative RT-PCR in vehicle- or E2-treated mHypoA-55 cells for 2 h or 4 h. Expression of *Rbbp7* was detected in the mHypoA-55 cells treated with vehicle, 10 nM or 100 nM E2 for 2 h, and no significant difference in *Rbbp7* expression between the groups was detected (Fig. 4A). On the other hand, 100 nM E2 treatment for 2 h tended to decrease *Kiss1* expression in the mHypoA-55 cells, compared with vehicle-treated cells (Fig. 4B). There was no significant difference in *Kiss1* mRNA levels at 4 h after treatment with vehicle, 10 nM or 100 nM E2 in the mHypoA-55 cells (Fig. 4C).

Figures 4 D–G show *Rbbp7* and *Kiss1* expression levels as determined by quantitative RT-PCR in the ARC and the AVPV regions obtained from OVX rats and low- or high-E2-treated OVX rats. Expression of *Rbbp7* was detected in both the ARC and the AVPV of the animals in all groups, and there was no significant difference in *Rbbp7* expression between the groups (Figs. 4D and 4E). On the other hand, *Kiss1* levels in the ARC of OVX rats were decreased by E2 treatment, in a dose-dependent manner, resulting in significant differences in *Kiss1* levels between OVX group and OVX + low E2 or OVX + high E2 groups (Fig. 4F; $P < 0.05$, one-way ANOVA with Bonferroni post-hoc test). E2 treatment increased *Kiss1* expression in the AVPV in a dose-dependent manner, resulting in significant difference in *Kiss1* level between OVX and OVX + high E2 groups (Fig. 4G; $P < 0.05$, one-way ANOVA with Bonferroni post-hoc test).

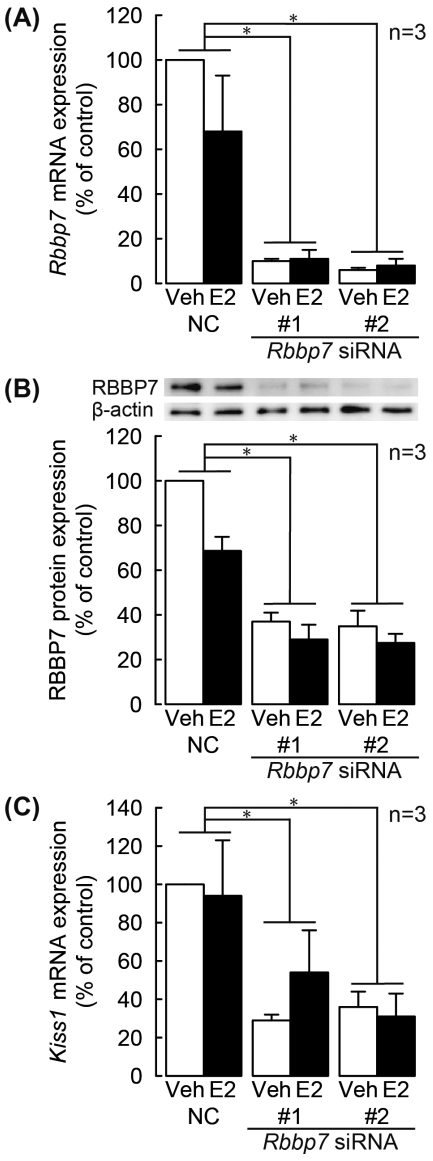


Fig. 3. Knockdown of retinoblastoma binding protein 7 (RBBP7) expression repressed *Kiss1* mRNA expression in the mHypo-A55 cells. *Rbbp7* mRNA expression in mHypoA-55 cells treated with E2 following *Rbbp7* siRNA transfection (A). NC, negative control siRNA; #1 and #2, two sets of siRNA against *Rbbp7*. The mRNA levels were measured by quantitative RT-PCR. Representative western blot bands and results of densitometric analysis of RBBP7 protein expression (B). The band intensities were estimated by densitometry using Image J. *Kiss1* mRNA expression in mHypoA-55 cells treated with E2 following *Rbbp7* siRNA transfection (C). Expression levels were normalized to *Actb* mRNA or β-actin protein. Data are the mean ± SEM (n = 3) and are expressed as % of controls treated with vehicle (Veh) and NC siRNA. * $P < 0.05$, two-way ANOVA with Bonferroni post-hoc test.

Discussion

The present study demonstrates that RBBP7 would be largely involved in the positive regulation of *Kiss1* mRNA expression in the hypothalamic kisspeptin neurons in rodents, because majority (more than 85%) of kisspeptin neurons in both the ARC and the AVPV regions expressed *Rbbp7* in female rats, and siRNA-mediated *Rbbp7* knockdown significantly decreased *Kiss1* expression in the mouse immortalized kisspeptin neuronal cell line. It should be noted that the *Rbbp7* knockdown reported in this study, resulted in a profound

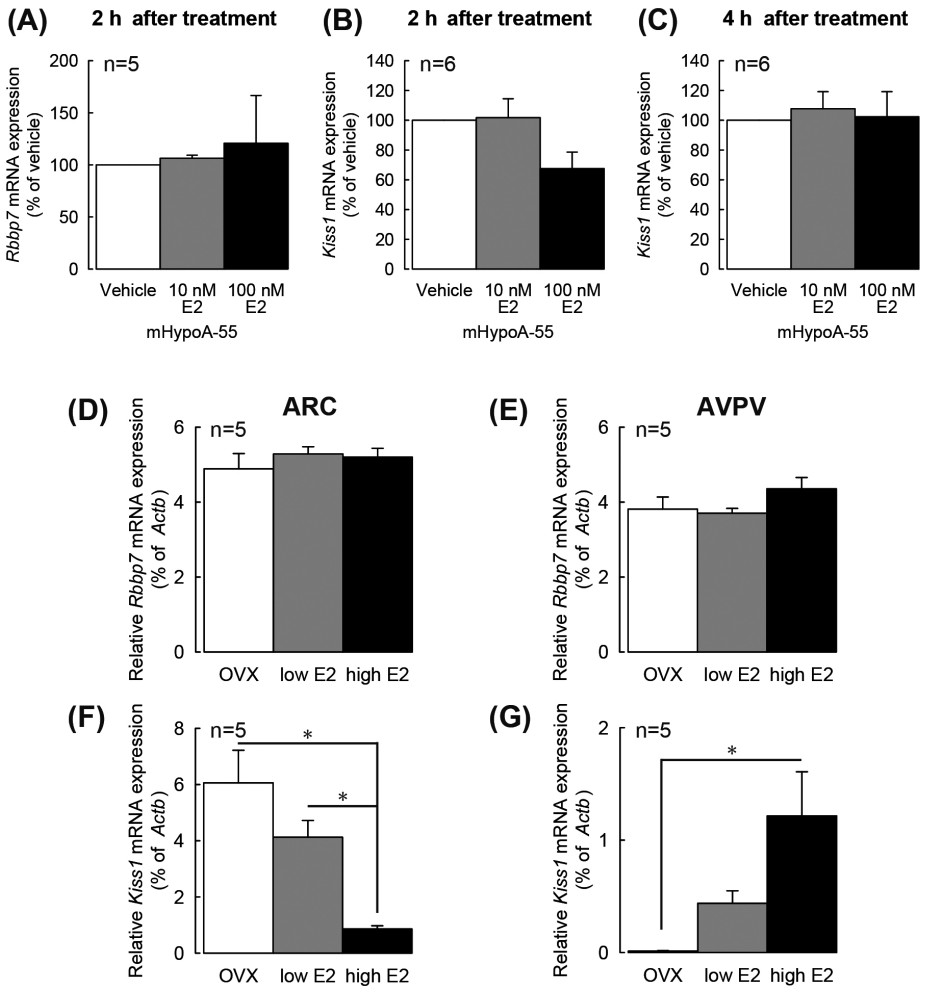


Fig. 4. *Rbbp7* and *Kiss1* expression in mHypoA-55 cells and in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) regions of ovariectomized (OVX) rats treated with E2. *Rbbp7* expression in mHypoA-55 cells treated with E2 for 2 h (A). *Kiss1* expression levels in mHypoA-55 cells treated with E2 for 2 h (B) and 4 h (C), respectively. Expression levels were normalized to *Actb* mRNA level. Data are the mean ± SEM (n = 5–6) and are expressed as % of vehicle-treated controls. Relative *Rbbp7* expression levels in the ARC (D) and AVPV (E) of OVX rats treated with diestrous levels (low) or proestrous levels (high) of E2. Relative *Kiss1* expression levels in the ARC (F) and AVPV (G) of OVX rats treated with low or high E2. The mRNA levels were measured by quantitative RT-PCR. Data are the mean ± SEM (n = 5–6) and are expressed as the relative ratio of *Rbbp7* or *Kiss1* mRNA to *Actb* mRNA. * $P < 0.05$, one-way ANOVA with Bonferroni post-hoc test.

decrease in the levels of RBBP7 protein. We had previously showed that histone acetylation of the *Kiss1* promoter region is positively associated with *Kiss1* expression in the ARC and the AVPV of female mice [27]. It was reported that *Rbbp7* silencing decreased histone H4K5, H4K8, H4K12 acetylation at the whole-genome level in the mouse stromal cells [42]. Thus, the *Rbbp7* mRNA silencing may attenuate histone acetylation of the *Kiss1* promoter and, subsequently, decrease *Kiss1* expression in kisspeptin neurons. Taken together, the present results suggest that RBBP7 could be involved in upregulation of *Kiss1* mRNA by affecting histone acetylation of *Kiss1* promoter region.

The present study showed that *in vivo* estrogen treatment at diestrous and proestrous levels failed to affect *Rbbp7* expression in the rat ARC and AVPV regions, although the estrogen treatments downregulated and upregulated *Kiss1* expressions in the ARC and the AVPV, respectively, in a dose-dependent manner. Further, *in vitro* estrogen treatment also failed to affect *Rbbp7* mRNA levels in the mHypoA-55 cells, a mouse ARC kisspeptin neuronal model. The present study showed that the *Rbbp7* knockdown largely suppressed *Kiss1* expression, compared to that in the NC siRNA-transfected cells, regardless of estrogen treatment. Taken together, these results suggest that RBBP7 mediates positive regulation of *Kiss1* expression in an estrogen-independent manner. Thus, RBBP7 may have a permissive role for the control of *Kiss1* levels, but not be involved in estrogen negative/positive feedback for *Kiss1* expression. In other words, RBBP7 could be constitutively involved in the upregulation of *Kiss1* expression, but not mediate the estrogen positive and negative feedback for *Kiss1* expression. Interestingly, previous studies reported that estrogen affects *Rbbp7* expression in a bidirectional manner:

estrogen increased *Rbbp7* mRNA and protein expression in MCF7 cells [45], whereas it inhibited *Rbbp7* expression in progression of transformed breast epithelial cells, MCF10AT3B cells [46]. Since our study implies that estrogen is unlikely to be involved in RBBP7 expression in the rodent hypothalamus, further studies are required to determine factor(s), mediating the estrogen positive/negative feedback for *Kiss1* expression, along with the factor(s), regulating *Rbbp7* and the protein expressions in hypothalamic kisspeptin neurons.

The current histological analysis with ISH for *Rbbp7* mRNA in the rat hypothalamus revealed that *Rbbp7* expression was located in several nuclei, such as the SCN, SON, PVN, and VMH in addition to the ARC and the AVPV. Considering the upregulating role of RBBP7 in *Kiss1* expression in our study, the widely-distributed RBBP7 in the hypothalamic nuclei implies that RBBP7 could also be involved in the gene expression of some neurotransmitters, such as arginine vasopressin (AVP) and vasoactive intestinal peptide in the SCN, oxytocin and AVP in the SON and PVN, corticotropin-releasing hormone in the PVN, and pituitary adenylate cyclase-activating polypeptide in the VMH [47, 48]. Further studies are required to clarify the role of RBBP7 in the gene expressions of these neurotransmitters in the nuclei.

The present study suggests that RBBP7 may act as a component of NuRD or HAT-B complexes in kisspeptin neurons, because the current RNA-seq data showed that major components of NuRD and HAT-B were detected in rat ARC kisspeptin neurons [35, 43]. It is unlikely that RBBP7 could act as a component of SIN3 and PRC2 complexes in kisspeptin neurons, because the RNA-seq data failed to detect major components of SIN3 and PRC2, such as *Sap30* and *Eed*, respectively [35, 44]. Previous reports [35, 43] suggest that NuRD serves as a major histone deacetylase complex in the nucleus, and HAT-B plays a role in the acetylation of histones H3 and H4 in newly-synthesized histones in cytoplasm. Thus, RBBP7, which may form the NuRD complex in the kisspeptin neurons, may repress the expression of some inhibitory gene(s) of *Kiss1* transcription, indirectly leading to an elevation of *Kiss1* expression. It would be interesting to examine whether RBBP7 and/or NuRD are involved in the pubertal increase in ARC *Kiss1* expression during peripubertal period. Further studies are needed to elucidate the mechanisms of RBBP7-mediated regulation of *Kiss1* expression in kisspeptin neurons.

In conclusion, the present findings suggest that RBBP7 is involved in the upregulation of *Kiss1* mRNA expression in kisspeptin neurons of rodents by increasing the histone acetylation level of *Kiss1* promoter region or repressing the expression of some inhibitory gene(s) for *Kiss1* transcription.

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References

1. **Uenoyama Y, Nakamura S, Hayakawa Y, Ikegami K, Watanabe Y, Deura C, Minabe S, Tomikawa J, Goto T, Ieda N, Inoue N, Sanbo M, Tamura C, Hirabayashi M, Maeda KI, Tsukamura H.** Lack of pulse and surge modes and glutamatergic stimulation of luteinising hormone release in *Kiss1* knockout rats. *J Neuroendocrinol* 2015; **27**: 187–197. [Medline] [CrossRef]
2. **d'Anglemont de Tassigny X, Fagg LA, Dixon JP, Day K, Leitch HG, Hendrick AG, Zahn D, Franceschini I, Caraty A, Carlton MB, Aparicio SA, Colledge WH.** Hypogonadotropic hypogonadism in mice lacking a functional *Kiss1* gene. *Proc Natl Acad Sci USA* 2007; **104**: 10714–10719. [Medline] [CrossRef]
3. **Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA.** Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci USA* 2005; **102**: 1761–1766. [Medline] [CrossRef]
4. **Topaloglu AK, Tello JA, Kotan LD, Ozbek MN, Yilmaz MB, Erdogan S, Gurbuz F, Temiz F, Millar RP, Yuksel B.** Inactivating *KISS1* mutation and hypogonadotropic hypogonadism. *N Engl J Med* 2012; **366**: 629–635. [Medline] [CrossRef]
5. **Tomikawa J, Homma T, Tajima S, Shibata T, Inamoto Y, Takase K, Inoue N, Ohkura S, Uenoyama Y, Maeda K, Tsukamura H.** Molecular characterization and estrogen regulation of hypothalamic *KISS1* gene in the pig. *Biol Reprod* 2010; **82**: 313–319. [Medline] [CrossRef]
6. **Inoue N, Sasagawa K, Ikai K, Sasaki Y, Tomikawa J, Oishi S, Fujii N, Uenoyama Y, Ohmori Y, Yamamoto N, Hondo E, Maeda K, Tsukamura H.** Kisspeptin neurons mediate reflex ovulation in the musk shrew (*Suncus murinus*). *Proc Natl Acad Sci USA* 2011; **108**: 17527–17532. [Medline] [CrossRef]
7. **Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS Jr, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF Jr, Aparicio SA, Colledge WH.** The GPR54 gene as a regulator of puberty. *N Engl J Med* 2003; **349**: 1614–1627. [Medline] [CrossRef]
8. **de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E.** Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA* 2003; **100**: 10972–10976. [Medline] [CrossRef]
9. **Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, Cerrato F, Le WW, Hoffman GE, Seminara SB.** *Kiss1*$^{-/-}$ mice exhibit more variable hypogonadism than *Gpr54*$^{-/-}$ mice. *Endocrinology* 2007; **148**: 4927–4936. [Medline] [CrossRef]
10. **Smith JT, Shahab M, Pereira A, Pau KY, Clarke IJ.** Hypothalamic expression of *KISS1* and gonadotropin inhibitory hormone genes during the menstrual cycle of a non-human primate. *Biol Reprod* 2010; **83**: 568–577. [Medline] [CrossRef]
11. **Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A.** Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett* 2006; **401**: 225–230. [Medline] [CrossRef]
12. **Matsuda F, Nakatsukasa K, Suetomi Y, Naniwa Y, Ito D, Inoue N, Wakabayashi Y, Okamura H, Maeda KI, Uenoyama Y, Tsukamura H, Ohkura S.** The luteinising hormone surge-generating system is functional in male goats as in females: involvement of kisspeptin neurones in the medial preoptic area. *J Neuroendocrinol* 2015; **27**: 57–65. [Medline] [CrossRef]
13. **Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA.** A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* 2004; **145**: 4073–4077. [Medline] [CrossRef]
14. **Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K.** Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev* 2007; **53**: 367–378. [Medline] [CrossRef]
15. **Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA.** Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* 2009; **29**: 11859–11866. [Medline] [CrossRef]
16. **Kinsey-Jones JS, Grachev P, Li XF, Lin YS, Milligan SR, Lightman SL, O'Byrne KT.** The inhibitory effects of neurokinin B on GnRH pulse generator frequency in the female rat. *Endocrinology* 2012; **153**: 307–315. [Medline] [CrossRef]
17. **Lehman MN, Coolen LM, Goodman RL.** Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* 2010; **151**: 3479–3489. [Medline] [CrossRef]
18. **Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, Wakabayashi Y, Uenoyama Y, Mori Y, Steiner RA, Tsukamura H, Maeda KI, Okamura H.** Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. *J Neuroendocrinol* 2009; **21**: 813–821. [Medline] [CrossRef]
19. **Clarkson J, Han SY, Piet R, McLennan T, Kane GM, Ng J, Porteous RW, Kim JS,**

Colledge WH, Iremonger KJ, Herbison AE. Definition of the hypothalamic GnRH pulse generator in mice. *Proc Natl Acad Sci USA* 2017; **114**: E10216–E10223. [Medline] [CrossRef]

20. **Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K.** Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. *Endocrinology* 2005; **146**: 4431–4436. [Medline] [CrossRef]
21. **Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M.** Sexual differentiation of *Kiss1* gene expression in the brain of the rat. *Endocrinology* 2007; **148**: 1774–1783. [Medline] [CrossRef]
22. **Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE.** Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci* 2008; **28**: 8691–8697. [Medline] [CrossRef]
23. **Homma T, Sakakibara M, Yamada S, Kinoshita M, Iwata K, Tomikawa J, Kanazawa T, Matsui H, Takatsu Y, Ohtaki T, Matsumoto H, Uenoyama Y, Maeda K, Tsukamura H.** Significance of neonatal testicular sex steroids to defeminize anteroventral periventricular kisspeptin neurons and the GnRH/LH surge system in male rats. *Biol Reprod* 2009; **81**: 1216–1225. [Medline] [CrossRef]
24. **Tsukamura H, Homma T, Tomikawa J, Uenoyama Y, Maeda K.** Sexual differentiation of kisspeptin neurons responsible for sex difference in gonadotropin release in rats. *Ann N Y Acad Sci* 2010; **1200**: 95–103. [Medline] [CrossRef]
25. **Uenoyama Y, Tomikawa J, Inoue N, Goto T, Minabe S, Ieda N, Nakamura S, Watanabe Y, Ikegami K, Matsuda F, Ohkura S, Maeda K, Tsukamura H.** Molecular and epigenetic mechanism regulating hypothalamic *Kiss1* gene expression in mammals. *Neuroendocrinology* 2016; **103**: 640–649. [Medline] [CrossRef]
26. **Semaan SJ, Kauffman AS.** Emerging concepts on the epigenetic and transcriptional regulation of the *Kiss1* gene. *Int J Dev Neurosci* 2013; **31**: 452–462. [Medline] [CrossRef]
27. **Tomikawa J, Uenoyama Y, Ozawa M, Fukanuma T, Takase K, Goto T, Abe H, Ieda N, Minabe S, Deura C, Inoue N, Sanbo M, Tomita K, Hirabayashi M, Tanaka S, Imamura T, Okamura H, Maeda K, Tsukamura H.** Epigenetic regulation of *Kiss1* gene expression mediating estrogen-positive feedback action in the mouse brain. *Proc Natl Acad Sci USA* 2012; **109**: E1294–E1301. [Medline] [CrossRef]
28. **Lomniczi A, Loche A, Castellano JM, Ronnekleiv OK, Bosch M, Kaidar G, Knoll JG, Wright H, Pfeifer GP, Ojeda SR.** Epigenetic control of female puberty. *Nat Neurosci* 2013; **16**: 281–289. [Medline] [CrossRef]
29. **Vazquez MJ, Toro CA, Castellano JM, Ruiz-Pino F, Roa J, Beiroa D, Heras V, Velasco I, Dieguez C, Pinilla L, Gaytan F, Nogueiras R, Bosch MA, Rønnekleiv OK, Lomniczi A, Ojeda SR, Tena-Sempere M.** SIRT1 mediates obesity- and nutrient-dependent perturbation of pubertal timing by epigenetically controlling *Kiss1* expression. *Nat Commun* 2018; **9**: 4194. [Medline] [CrossRef]
30. **Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y.** Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 2002; **298**: 1039–1043. [Medline] [CrossRef]
31. **Simon JA, Kingston RE.** Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat Rev Mol Cell Biol* 2009; **10**: 697–708. [Medline] [CrossRef]
32. **Verreault A, Kaufman PD, Kobayashi R, Stillman B.** Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell* 1996; **87**: 95–104. [Medline] [CrossRef]
33. **Philpott A, Krude T, Laskey RA.** Nuclear chaperones. *Semin Cell Dev Biol* 2000; **11**: 7–14. [Medline] [CrossRef]
34. **Parthun MR.** Histone acetyltransferase 1: More than just an enzyme? *Biochim Biophys Acta* 2012; **1819**: 256–263. [Medline] [CrossRef]
35. **Ahringer J.** NuRD and SIN3 histone deacetylase complexes in development. *Trends Genet* 2000; **16**: 351–356. [Medline] [CrossRef]
36. **Assadullah IN, Ieda N, Kawai N, Ishii H, Ihara K, Inoue N, Uenoyama Y, Tsukamura H.** Co-expression of the calcitonin receptor gene in the hypothalamic kisspeptin neurons in female rats. *Reprod Med Biol* 2018; **17**: 164–172. [Medline] [CrossRef]
37. **Cagampang FR, Maeda KI, Tsukamura H, Ohkura S, Ota K.** Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH release in ovariectomized rats. *J Endocrinol* 1991; **129**: 321–328. [Medline] [CrossRef]
38. **Paxinos G, Watson C.** The Rat Brain in Stereotaxic Coordinates, 5th Edition. San Diego: Elsevier Academic Press; 2005.
39. **Sugimoto A, Tsuchida H, Ieda N, Ikegami K, Inoue N, Uenoyama Y, Tsukamura H.** Somatostatin-somatostatin receptor 2 signaling mediates LH pulse suppression in lactating rats. *Endocrinology* 2019; **160**: 473–483. [Medline] [CrossRef]
40. **Treen AK, Luo V, Chalmers JA, Dalvi PS, Tran D, Ye W, Kim GL, Friedman Z, Belsham DD.** Divergent regulation of ER and Kiss genes by 17β-estradiol in hypothalamic ARC versus AVPV models. *Mol Endocrinol* 2016; **30**: 217–233. [Medline] [CrossRef]
41. **Takase K, Uenoyama Y, Inoue N, Matsui H, Yamada S, Shimizu M, Homma T, Tomikawa J, Kanda S, Matsumoto H, Oka Y, Tsukamura H, Maeda KI.** Possible role of oestrogen in pubertal increase of *Kiss1*/kisspeptin expression in discrete hypothalamic areas of female rats. *J Neuroendocrinol* 2009; **21**: 527–537. [Medline] [CrossRef]
42. **He H, Kong S, Liu F, Zhang S, Jiang Y, Liao Y, Jiang Y, Li Q, Wang B, Zhou Z, Wang H, Huo R.** Rbbp7 is required for uterine stromal decidualization in mice. *Biol Reprod* 2015; **93**: 13. [Medline] [CrossRef]
43. **Parthun MR.** Hat1: the emerging cellular roles of a type B histone acetyltransferase. *Oncogene* 2007; **26**: 5319–5328. [Medline] [CrossRef]
44. **Margueron R, Reinberg D.** The Polycomb complex PRC2 and its mark in life. *Nature* 2011; **469**: 343–349. [Medline] [CrossRef]
45. **Creekmore AL, Walt KA, Schultz-Norton JR, Ziegler YS, McLeod IX, Yates JR, Nardulli AM.** The role of retinoblastoma-associated proteins 46 and 48 in estrogen receptor α mediated gene expression. *Mol Cell Endocrinol* 2008; **291**: 79–86. [Medline] [CrossRef]
46. **Zhang TF, Yu SQ, Wang ZY.** RbAp46 inhibits estrogen-stimulated progression of neoplastigenic breast epithelial cells. *Anticancer Res* 2007; **27**(5A): 3205–3209. [Medline]
47. **Tohyama M, Takatsuji K.** Atlas of Neuroactive Substances and Their Receptors in the Rat. Oxford: Oxford University Press; 1998: 226–317.
48. **Dürr K, Norsted E, Gömüç B, Suarez E, Hannibal J, Meister B.** Presence of pituitary adenylate cyclase-activating polypeptide (PACAP) defines a subpopulation of hypothalamic POMC neurons. *Brain Res* 2007; **1186**: 203–211. [Medline] [CrossRef]