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Circadian transcriptional factor DBP regulates expression of *Kiss1* in the anteroventral periventricular nucleus

Zhifang Xu [a], Shigehito Kaga [a], Jun Tsubomizu [a], Jun Fujisaki [a], Akikazu Mochiduki [a,1], Takafumi Sakai [a], Hiroko Tsukamura [b], Kei-ichiro Maeda [b], Kinji Inoue [a], Akihito A. Adachi [a,*]

[a] *Graduate School of Science and Engineering, Saitama University, Saitama 338-8570, Japan*
[b] *Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*

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ABSTRACT

The expression of *Kiss1* in the anteroventral periventricular nucleus (AVPV) and its product, metastin/kisspeptin, show a circadian pattern with a peak in the evening, which shows a strong phase relationship with the time of the gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) surge in rodents. Here we report that a circadian transcriptional factor, albumin D-site binding protein (*Dbp*), was able to trigger *mKiss1* transcription via the D-box, and this effect was combined with those of estrogen receptor α (ERα) and its ligand, estrogen. A histological study demonstrated that some cells in the AVPV co-expressed *Dbp* with ERα in adult female rats. Expression of ERα was not rhythmic in the AVPV, however, mRNA of *Dbp* in the AVPV accumulated with a robust diurnal rhythm in proestrus, but not on the first day of diestrus. Thus, these results suggest that *Dbp* and estrogen regulate the expression of *Kiss1* in the AVPV, thereby mediating the GnRH/LH surge.

1. Introduction

One of the critical sexual differentiations in mammals reflects the presence of estrogen-inducing positive feedback in females, which is regulated by the hypothalamic–pituitary–gonadal (HPG) axis. During the late afternoon of proestrus in adult female rodents, a sharp increase in serum estrogen secreted by dominant follicles of the ovaries elicits an acute increase in the GnRH pulse frequency, which generates a LH surge that triggers ovulation (Gottsch et al., 2004; Christian et al., 2005). GnRH secretion is mediated by ERα (Wintermantel et al., 2006), however, there is no expression of ERα in GnRH neurons (Herbison and Pape, 2001).

Since metastin/kisspeptin was isolated from a human placenta and identified as a natural ligand of G-protein-coupled receptor 54 (*GPR54*) (also known as *Kiss1r*) (Ohtaki et al., 2001; Kotani et al., 2001; Muir et al., 2001), a series of studies has established that metastin/GPR54 is the link between estrogen/ERα and GnRH neurons (Seminara et al., 2003; Maeda et al., 2007). Firstly, *Kiss1* and *Gpr54* knockout females exhibit hypogonadotropic hypogonadism, which includes the absence of an estrous cycle (de Roux et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007). Secondly, metastin/kisspeptin neurons in the AVPV project axonal fibers to GnRH neurons (Clarkson and Herbison, 2006), which also express *Gpr54* (Han et al., 2005). Thirdly, not only do *Kiss1* neurons in the AVPV express ERα (Smith et al., 2005; Adachi et al., 2007), but also estrogen up-regulates *Kiss1* expression *in vivo* and through ERα binding to estrogen response element (ERE) sites of its promoter *in vitro* (Li et al., 2007; Gottsch et al., 2009). Moreover, *Kiss1* expression in the AVPV increases just before the LH surge occurs (Smith et al., 2006; Robertson et al., 2009). It is most conclusive that we demonstrated that the infusion of metastin/kisspeptin antibodies prevented the LH surge in rats (Kinoshita et al., 2005; Adachi et al., 2007). Thus, *Kiss1* plays a major role in the induction of the GnRH/LH surge.

The GnRH/LH surge is gated by the biological clock in some rodent species. The surge event is precisely timed to occur in a narrow 2–4 h window before darkness onset. In normal female rodents, the LH surge is fixed in the afternoon of proestrus as estrogen is high on that day. However, the LH surge occurs daily in the evening in

Abbreviations: AVPV, anteroventral periventricular nucleus; ccg, clock-controlled gene; AVP, arginine vasopressin; *Cry*/CRY, cryptochrome; *Dbp*/DBP, albumin D-site binding protein; ERα, estrogen receptor α; ERβ, estrogen receptor β; ERE, estrogen response element; FBS, fetal bovine serum; GnRH, gonadotropin-releasing hormone; GPR54, G-protein-coupled receptor 54; *Hlf*, hepatic leukemia factor; HPG axis, hypothalamic–pituitary–gonadal axis; IF, immunofluorescence; ISH, *in situ* hybridization; LH, luteinizing hormone; OVX, ovariectomized; *Per*, *Period*; RRE, Rev-erb/Ror element; SCN, suprachiasmatic nucleus; *Tef*, thyrotroph embryonic factor; ZT, zeitgeber time.

* Corresponding author at: Department of Regulatory Biology, Faculty of Science, Saitama University, 255 Shimo-Okubo, Sakuraku, Saitama 338-8570, Japan. Tel.: +81 48 858 3423; fax: +81 48 858 3423.
E-mail address: akihito@mail.saitama-u.ac.jp (A.A. Adachi).
[1] Present address: Department of Molecular Pharmacology, National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan.

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ovariectomized (OVX) female rodents with a stable high estrogen level (de la Iglesia and Schwartz, 2006). As a central master clock of the circadian system, the suprachiasmatic nucleus (SCN) possibly facilitates and sets the time of the LH surge, as SCN lesions and mutation of clock genes abolish estrogen implant, triggering a daily surge (Brown-Grant and Raisman, 1977; Miller et al., 2004, 2006). Our previous data and other studies clearly demonstrated that the level of *Kiss1* mRNA in the AVPV is higher in the late afternoon of proestrus in normal female and in the daily evening in estrogen-treated OVX females (Smith et al., 2006; Adachi et al., 2007; Robertson et al., 2009). Therefore, metastin/kisspeptin cells in the AVPV may act as a link between the circadian rhythm and GnRH neurons. However, the potential intrinsic mechanism of circadian regulation of *Kiss1* expression remains to be elucidated in detail.

Several lines of evidence have suggested that the daily rhythm of *Kiss1* could be controlled by arginine vasopressin (AVP) from the SCN (Zhao and Kriegsfeld, 2009). The expression of AVP in the SCN is regulated by the core circadian clock genes *Bmal1* and *Clock* directly (Grace et al., 1999; Munoz et al., 2002; Lowrey and Takahashi, 2004). Metastin/kisspeptin neurons in the AVPV are innervated by AVP immunoreactive fibers (Vida et al., 2010). Furthermore, the administration of AVP triggers the LH surge in SCN-lesioned, OVX + estrogen-treated females (Miller et al., 2006). However, if the signal for the LH surge from the SCN is only dependent on AVP release, the administration of AVP should induce the surge at any time of the day, although Palm et al. (2001) have shown a time-dependent effect of AVP administration on the LH surge. Therefore, it is possible that interneurons and/or *Kiss1*-positive neurons themselves possess a circadian system to regulate the LH surge with the gating effect of the administration.

The molecular mechanism of the circadian clock in mammals involves some interlocking positive and negative transcriptional and translational feedback loops of clock genes. In the primary feedback loop, the transcription of *Period* genes (*Per1* and *Per2*) (Hastings et al., 1999; Matsui et al., 2005) and cryptochromes (*Cry1* and *Cry2*) is triggered by bHLH transcriptional factors, *Bmal1* and *Clock* (Kume et al., 1999). The PER and CRY proteins repress their own transcription by acting on the BMAL1: CLOCK heterodimer (Jin et al., 1999; Kume et al., 1999). Besides this main feedback loop, there are another two loops, one involving the two retinoic acid-related orphan receptors *Nr1d1* and *Rora* (also called *RevErbAα* and *Rorα*) via the Rev-erb/Ror element (RRE), and the other comprising bZip-family genes (*Dbp* and *E4bp4*) via the D-box (Wuarin and Schibler, 1990; Mitsui et al., 2001; Preitner et al., 2002; Sato et al., 2004). These three loops not only modulate the phases of expression of these genes via the E/E′-box, RRE and D-box, but also regulate other cyclic genes (such as clock controlled gene ccg) (Ueda et al., 2005).

In this study, we hypothesized that *Kiss1*-positive neurons exhibit an intrinsic circadian system, and we examined the regulation of clock genes and clock-related genes, which are included in loops other than the core bHLH loop, in the time-dependent transactivation of *Kiss1*, and the molecular links between these genes and the positive feedback effect of estrogen.

2. Materials and methods

2.1. Chemicals and constructs

A PCR fragment of mouse *Kiss1* (*mKiss1*) promoter (−5280 to +41, 5.3 kbp) was obtained from BAC clone RP24-299J2 using the primers below, and the fragment was subcloned into the pENTR™/D-TOPO vector (Invitrogen, CA, USA). The −2715 to 41 bp (2.8 kbp) and −1215 to 41 bp (1.3 kbp) promoter plasmids were generated by digesting the 5.3 kbp *mKiss1* promoter with NotI and HindIII, and NotI and SwaI, respectively, followed by blunt-end ligation. After sequencing to confirm the inserted fragments of *mKiss1* promoters, the LR recombination reaction was carried out for ligation into the Gateway-designated pGL3-enhance vectors (Promega, WI, USA), named pGL3-*mKiss1*-5.3, pGL3-*mKiss1*-2.8, and pGL3-*mKiss1*-1.3. PCR-based mutagenesis was performed to construct a pGL3-*mKiss1*-5.3ΔD plasmid with the DBP binding site deleted from pGL3-*mKiss1*-5.3 and sequencing to confirm the deletion. The primers used for subcloning and for deleting mutations were as follows:

pGL3-*mKiss1*-5.3:
Forward: 5′-GCTGTGAATGTATCTGAGATTTGGCGTTGA-3′,
Reverse: 5′-CATTCTGGGAGGAAGGGGCAGGTCTACAGT-3′.

pGL3-*mKiss1*-5.3ΔD:
Forward: 5′-CTGCCCAGCACATCTGGCCGTGTCATATCA-3′,
Reverse: 5′-GCAGCTGGCTGTTAGGA AGGAGGGCAAAGC-3′.

The expression constructs were made as follows. The coding regions of *Dbp*, *Rorα*, and *Rev-erbα* were obtained by reverse transcription-PCR from mouse liver mRNA and used after confirming their sequences. The mouse *Clock* and *Bmal1* expression vectors were kindly provided by Dr. Hajime Tei. The rat ERα plasmid (pUC-ER6 GeneID: 2490) was obtained from the Riken Bioresource Center with the permission of Dr. Masami Muramatsu.

2.2. Cell culture, transfection and dual-luciferase report assays

Cos-7 cells were maintained in 96-well plates in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA, USA) at 37 °C 5% CO_2. The cell number in each well was 3×10^4/100 μl 24 h before transfection. The reporter gene plasmids and the phRL-TK vector (Promega, WI, USA) as an internal control were transfected together with various expression constructs by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. pcDNA 3.1 (Invitrogen, CA, USA) was added to adjust the DNA concentration to 180–300 ng/well. Four hours later, the medium was replaced with 17β-estradiol (E_2; Sigma Chemicals, MO, USA) or 0.1% ethanol as a control in DMEM (10% FBS). Thirty-six hours after transfection, cells from different treatment groups were obtained by passive lysis, and then luciferase activity was measured by using a Dual-luciferase reporter assay system ($n = 3$; Promega, WI, USA).

2.3. Animals and treatments

For morphological analyses, adult female Wistar rats (10–12 weeks old, 250–300 g) were housed in a controlled environment (12 h light and 12 h darkness, lights on at 8:00; temperature, 21–24 °C; humidity, 60%), and allowed free access to standard laboratory rat chow and water *ad libitum*. Vaginal smears were checked daily to determine the stage of the estrous cycle (proestrus, estrus, diestrus1, and diestrus2), rats with two consecutive estrous cycles being used. The morphological studies were conducted in accordance with Saitama University institutional guidelines for animal care.

2.4. Tissue preparations

Rats were deeply anesthetized and perfused with 4% paraformaldehyde in 0.07 M phosphate buffer (PH 7.4) at four time points, diestrus1-ZT21 (zeitgeber time 21, actual time 5:00), diestrus1-ZT9 (actual time 17:00), proestrus-ZT21 (P-ZT21, the anti-phase of P-ZT9), and proestrus-ZT9. Their brains were immediately removed, post-fixed in the same fixative for 16 h at 4 °C, immersed in PBS containing 30% sucrose for 72 h at 4 °C and then frozen in an optimal cutting temperature compound (Sakura Finetek, CA, USA) with liquid nitrogen maintained at −80 °C.

2.5. Single-label in situ hybridization (ISH)

We made *Dbp* (positions 508–1298; GenBank accession no. 13170) and *Kiss1* (positions 33–348; GenBank accession no. AY196983) specific digoxigenin (Dig)-labeled probes, and performed non-radioactive free-floating *in situ* hybridization to detect *Dbp* and *Kiss1* mRNA. The *in situ* hybridization method was used as previously described (Adachi et al., 2007). *Dbp* mRNA detection in the SCN was performed as a positive control and detection with a sense probe as a negative control.

2.6. Double staining for in situ hybridization (ISH) and immunofluorescence (IF)

We performed double staining to determine whether or not *Dbp* mRNA-positive cells possess ERα and to confirm that *Kiss1*-positive neurons co-express ERα. After *in situ* hybridization without ProK treatment, free-floating sections (45 μm) were washed with PBS. The sections were then incubated in 1% normal fetal bovine serum (FBS) and 0.1% Triton X-100 in PBS for 1 h. After incubation with rabbit anti-ERα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:1000) in 1% FBS for 14 h, the sections were treated with Alexa 594-conjugated donkey anti-rabbit IgG (10 μg/ml; Molecular Probes Company, Inc., OR, USA) in 1% FBS for 2 h. Finally, all the sections were mounted in rostral-caudal sequence, and observed by light microscopy and fluorescence microscopy.

2.7. Quantification of single and dual-labeled staining

Analysis was performed by counting the *Dbp* mRNA-positive neurons in the AVPV. The counting area was determined by placing a rectangle (140 μm × 400 μm) next to the third ventricle, which is a *Kiss1*-expressing region, at the level of Fig. 19