Rapid modulation of hypothalamic Kiss1 levels by the suckling stimulus in the lactating rat

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Short title: Suckling acutely modulates hypothalamic Kiss1.

Key words: Lactation, Reproduction, Gonadotrophin releasing hormone, Prolactin

Word count: 4781
Abstract

In mammals, lactation suppresses GnRH/LH secretion resulting in transient infertility.

In rats, GnRH/LH secretion is rescued within 18–48 h after pup separation (PS) and rapidly re-suppressed by subsequent re-exposure of pups. To elucidate the mechanisms underlying these rapid modulations, changes in the expression of kisspeptin, a stimulator of GnRH secretion, in several lactating conditions (normal-lactating; 4-h PS; 18-h PS; 4-h PS + 1-h re-exposure of pups; non-lactating) were examined using in situ hybridization. PS for 4 h or 18 h increased Kiss1 expressing neurons in both the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC), and subsequent exposure of pups re-suppressed Kiss1 in the AVPV. A change in Kiss1 expression was observed prior to the reported time of the change in GnRH/LH, indicating that the change in GnRH/LH results from changes in kisspeptin. We further examined the mechanisms underlying the rapid modulation of Kiss1. We first investigated the possible involvement of ascending sensory input during the suckling stimulus. Injection of the anterograde tracer to the subparafascicular parvocellular nucleus (SPFpc) in the midbrain, which relays the suckling stimulus, revealed direct neuronal connections between the SPFpc and kisspeptin neurons in both the AVPV and ARC. We also examined the possible involvement of prolactin. Administration of
prolactin for 1 h suppressed Kiss1 expression in the AVPV but not in the ARC. These results indicate that suckling stimulus rapidly modulates Kiss1 expression directly via neuronal connections and indirectly through serum prolactin, resulting in modulation in GnRH/LH secretion.
Introduction

Lactation suppresses ovulation resulting in transient infertility in various mammals including humans (McNeilly 2001). Lactation-induced infertility is characterized by a decrease in GnRH secretion from the hypothalamus, and a consequent decrease of luteinizing hormone (LH) secretion from the pituitary (Fox and Smith 1984; Maeda, et al. 1987; Smith and Neill 1977). Several studies indicate that LH secretion is dynamically modified along with the modulation of the suckling stimulus in a rapid manner; in rats, Maeda et al. showed that lactation-induced decreases in GnRH/LH secretion are rescued in 18–48 h after pup-separation (PS) and are rapidly re-suppressed by subsequent re-exposure of pups (Maeda, et al. 1989); Li et al. also reported the increase of plasma LH by 48-h PS and re-suppression by 24-h re-exposure of pups (Li, et al. 1998); and Fukushima et al. showed that estrogen responsiveness of LH secretion was restored within 6 h after PS (Fukushima, et al. 2006). Although these studies suggest that the proximal cause of these rapid changes in GnRH/LH secretion is the suckling stimulus, the neuroendocrine mechanisms linking the modulation in suckling stimulus and the change in GnRH/LH secretion are not yet fully understood.

Recent studies have shown that kisspeptin, a product of the Kiss1 gene, plays an important role in regulating GnRH/LH secretion via activation of the G-protein coupled
receptor 54 (GPR-54) in various animals (Kotani, et al. 2001). In rodents, there are two
discrete subpopulations of kisspeptin immunoreactive neurons with different
physiological functions; the anteroventral periventricular nucleus (AVPV) and the
arcuate nucleus (ARC) in the hypothalamus. With regard to its physiological function,
kisspeptin neurons in the AVPV and ARC are responsible for the generation of surge
and pulse patterns in GnRH/LH secretion, respectively (Li, et al. 2009; Smith, et al.
2006b). In non-pregnant and non-lactating rodents, kisspeptin in the AVPV and ARC are
regulated by estrogen in an opposite manner. In particular, kisspeptin neurons in the
AVPV are positively regulated by estradiol, and those in the ARC are negatively

Several studies indicate the importance of kisspeptin systems in the lactation-induced
al. 2011; Yamada, et al. 2012). For example, *Kiss1* mRNA and kisspeptin peptide are
reduced in the AVPV and ARC of lactating mice compared with non-lactating mice (Liu
et al. 2014). In ovariectomized (OVX) rats, lactation induces the suppression of *Kiss1*
mRNA expression in the ARC, and reduced responsiveness of kisspeptin synthesis to
estradiol in the AVPV (Yamada et al. 2012; Yamada, et al. 2007). However, despite
these studies, information about kisspeptin synthesis following modification of the
suckling stimulus (PS and re-suckling condition) remains incomplete.

The aim of this study is to clarify whether the change in kisspeptin system underlies the rapid change of LH secretion after the modification of suckling conditions, and to investigate the cellular mechanisms of the change. We examined the change in both Kiss1 mRNA and the kisspeptin peptide in lactating rats under PS and re-suckling conditions using in situ hybridization and immunohistochemistry. We also investigated possible mechanisms linking the suckling stimulus and Kiss1 mRNA expression. We first showed significant changes in Kiss1 mRNA expression in the AVPV and the ARC along with manipulation of lactating conditions. Second, we showed direct neuronal connections between the subparafascicular parvocellular nucleus (SPFpc), which is responsible for relaying the suckling stimulus and kisspeptin neurons in both the AVPV and the ARC using a anterograde tracing combined with immunohistochemistry against kisspeptin. Finally, we examined the involvement of serum prolactin (PRL) on the change in Kiss1 mRNA expression.

**Materials and Methods**

**Animals**

Pregnant Wistar rats and 10-week-old female Wistar rats were purchased from Tokyo
Laboratory Animals Science (Tokyo, Japan). Pregnant rats were mated between 8 to 10 weeks of age. Rats were housed on a 14-h/10-h light/dark cycle (light on from 06:00 to 20:00), with unrestricted access to food and water. All experiments were performed in accordance with the institutional guidelines for the use of experimental animals, and were approved by the Ethics Committee of Nippon Medical School.

Manipulation of lactation condition

The day newborn pups were discovered before noon was designated day 0 of lactation. The litter size was adjusted to 8 on Day 1. Some rats were separated from their pups in the afternoon of day0 and served as non-lactating controls. Maeda et al reported that LH secretion took at least 12h to recover after pup separation (PS), and that 6h PS was insufficient for recovery (Maeda et al. 1989). In this study, we used a short 4h PS protocol in addition to 18h PS, to clarify whether the change in kisspeptin preceded the recovery of LH secretion. Pregnant dams were separated into five groups: normal lactating (n=8); PS for 4h before sampling (n=6); PS for 4h followed by re-suckling for 1h (n=5); PS for 18h (n=6); non-lactating control (n=7). Cytology of vaginal smears was examined from day 4 until the day of sampling. Brain and blood sampling was performed between 11:00 and 14:00 on day 8. Lactating conditions and
the timeline of sampling are summarized in Fig. 1.

In situ hybridization

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and transcardially perfused with 50 mL of saline followed by 200 mL of fixative containing 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). Brains were quickly removed and postfixed in the same fixative for 24 h at 4°C, and transferred into phosphate buffered saline (PBS; 0.1M phosphate buffer, 0.9% (w/v) NaCl, pH 7.4) containing 20% (w/v) sucrose for cryoprotection. Four series of serial coronal sections at 30-µm thickness were cut using a cryostat (Leica 3050, Heidelberg, Germany), and collected into diethylpyrocarbonate (DEPC)-treated PBS. One series of sections containing seven sections of the AVPV (approx. AP 0.15 to -0.60 mm from Bregma), and 16 or 17 sections of the ARC (approx. AP -1.70 to -4.00 mm from Bregma) were used. Sections were treated with 1 µg/mL proteinase K (Takara Bio Inc., Otsu, Japan) in 10 mM Tris buffer (pH 7.4) and 10 mM EDTA for 15 min at 37°C. After two washes in PBS, sections were incubated with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine for 20 min at room temperature. After the prehybridization treatment in prehybridization buffer (1×hybridization solution (Sigma-Aldrich, St Louis, MO, USA)
containing 50% (v/v) formamide and 10% (w/v) dextran sulfate), sections were hybridized with 1 ng/mL digoxigenin (DIG) -labeled RNA probes for Kiss1 diluted in prehybridization buffer for 16 h at 60°C. Anti-sense and sense RNA probes were synthesized from template cDNA of rat full-length rat Kiss1 (GeneBank accession #AY196983) (Terao, et al. 2004) using a DIG-RNA labeling kit (Roche Diagnostics, Mannheim, Germany). After hybridization, sections were rinsed in 4X saline sodium sitrate (SSC) containing 50% formamide followed by 2X SSC for 20 min at 60°C, and treated with RNaseA (20µg/mL in 10 mM Tris-HCl (pH 8.0), 1mM EDTA, and 500 nM NaCl) for 20 min at 37°C. Sections were then washed under conditions of increasing stringency. DIG-labeled RNA probes were visualized using standard immunohistochemical procedures with the alkaline phosphatase conjugated anti-DIG antibody (Roche Diagnostics) and chromogen solution containing nitro blue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche Diagnostics) in Tris-HCl buffered saline (pH 9.5). Color development was performed in a dark box for 3 h at 25°C, and was ceased by several washes in PBS. Images of each section were acquired using a BX-51 microscope (Olympus). Kiss1 mRNA expressing neurons in each section were counted using ImageJ (v1.47, NIH, USA) with the Cell Counter plugin.
Immunohistochemistry

Immunohistochemistry against kisspeptin was performed using a streptavidin-horseradish peroxidase (HRP) based kit (Histofine SAB-PO kit, Nichirei Corporation, Tokyo, Japan). A series of coronal sections from each animal (seven sections of the AVPV, and 16 or 17 sections of the ARC) were treated with 0.03% (v/v) H$_2$O$_2$ in PBS to quench endogenous peroxidase activity, and then washed three times in PBS. After blocking in 5% (v/v) normal rabbit serum, sections were immersed in anti-kisspeptin monoclonal antibody (a kind gift from Takeda Pharmaceutical Co. Ltd, Tokyo, Japan) solution at a dilution of 1:2000 in PBS and incubated for 48 h at 4°C. The specificity of the antibody was reported previously (Kinoshita, et al. 2005). Kisspeptin neurons were visualized broadly following the protocol supplied by the manufacturer of the Histofine kit as described in our previous research (Iijima, et al. 2011). Kisspeptin visualization was achieved with diaminobenzidine (DAB; DAKO Inc. Carpinteria, CA, USA). Kisspeptin-positive neurons in each section were counted using ImageJ.

Anterograde tracer injection and immunohistochemistry

Injection of the primary anterograde tracer biotinylated dextran amine (BDA, MW
10,000, Life Technologies, Carlsbad, CA, USA) was performed according to Szabo et al. (Szabo, et al. 2010) with slight modification using a stereotaxic instrument (SR-5R, Narishige, Tokyo, Japan). Rats were anesthetized with 2.5% (v/v) isoflurane during stereotaxic surgery. A 0.05 µL of 5% (w/v) BDA in saline was injected unilaterally into the SPFpc by pressure through a glass micropipette attached to a Picospritzer III (General Valve Corporation, NJ, USA), using the following coordinates; AP −4.9; ML 2.7 in relation to bregma; DV 6.9 from skull surface. Forty-eight hours after BDA injection, colchicine (50 µg/10 µL saline, Wako Chemicals, Tokyo, Japan) was administered intracerebroventricularly (stereotaxic coordinates: AP −1.2; ML 2.2, DV 3.6) to enhance the immunoreactivity for the kisspeptin, using a Hamilton syringe (10 µL). Twenty-four hours after colchicine injection, rats were perfusion fixed using fixative containing 4% (w/v) PFA, and 0.25% (w/v) glutaraldehyde in 0.1 M PB. Brains were postfixed in 4 % PFA for 24 h at 4°C and cryoprotected in PBS containing 20% (w/v) sucrose. Serial coronal sections at 30-μm thickness were cut using a cryostat, and collected into PBS. Simultaneous visualization of BDA-positive projections and kisspeptin neurons was performed using a Histofine SAB-PO kit (Nichirei Corporation). After quenching endogenous peroxidase in 0.03% (v/v) H₂O₂, sections were incubated in streptavidin-HRP solution (Histofine SAB-PO kit) for 1h at room temperature. After
washing in PBS, BDA was visualized using a chromogen solution containing DAB, nickel sulfite, and cobalt chloride with H₂O₂ in 0.05M Tris-HCl buffer (pH 7.5). After quenching residual HRP activity in 0.3% (v/v) H₂O₂, normal immunohistochemical procedures were performed as described above, with DAB as a chromogen. Images of each section were acquired using a BX-51 microscope (Olympus). We used 10×, 20×, and oil immersion 100× objective lenses for acquisition of low-, medium-, and high-magnification images, respectively. Only those animals in which the injection site was located in the targeted areas (four animals, two parous and two non-parous) were included in the results.

Prolactin assay

Blood samples were collected from the right ventricle of the heart just before perfusion under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneally) and mixed with aprotinin (Takara Bio) at a final concentration of 50 µg/mL. Blood collection of was performed within 4-6 min after pentobarbital injection. Prolactin (PRL) levels were measured using a rat PRL ELISA Kit (CUSABIO, Hubei, China). After incubation for 30 min at room temperature, blood samples were centrifuged at 1600 × g for 15 min at 4°C, and the supernatant was collected in plastic tubes. All the
samples were stored at −80°C and assayed simultaneously. The hormone concentrations in each sample were assessed according to the manufacture’s specifications. A calibration curve was calculated according to the Rodbard model (DeLean, et al. 1978). Samples were diluted 1/5, and the assay was performed in duplicate.

Continuous administration of prolactin

Two days before sampling, a catheter made of silicone rubber tubing (SILASCON 100N-00, Kaneka Medical Product, Osaka, Japan) was inserted through the right jugular vein into the right atrium of the lactating rat under anesthesia using 2.5% (v/v) isoflurane. The catheter was filled with 40% (v/v) polyvinylpyrrolidone in heparinized saline until PRL administration. Before sampling (18h), pup separation and injection of bromocriptine (1mg/kg, subcutaneously) were performed. PRL (ovine prolactin, Sigma-Aldrich,) solution (9.0 mg/mL in saline, n=5) or saline (for control, n=4) was continuously administered through the catheter for 60 min at a flow rate of 1 µL/min. The dose of PRL was comparable to that used in previous reports (Araujo-Lopes et al. 2014; Bridges and Ronsheim 1990). Immediately after cessation of PRL administration, the rats were perfusion-fixed and sampled. Kiss1 mRNA expression in each sample was assessed by in situ hybridization as described above.
Statistical analysis

One-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test was applied for the analysis of Kiss1 mRNA expression, kisspeptin immunoreactivity, and serum PRL concentrations in manipulated lactation conditions. The Students’ t-test was performed for analysis of Kiss1 mRNA expression in continuous PRL administration. Statistical analyses were performed using IBM SPSS statistics software, with a p-value of less than 0.05 considered statistically significant.

Results

Cytology of vaginal smears in lactating rats

No ovarian cycle was observed in the cytology of the vaginal smears in normal lactating, 4-h PS, re-suckling after 4-h PS, and 18-h PS groups. The cytology of vaginal smears in all groups were displayed characteristics similar to those during diestrus. In non-lactating groups, although the cytology was relatively obscurer compared with normal diestrus rats, the vaginal cytology on the day of sampling from one rat was similar to that of proestrus, two were similar to that of estrus, and four were similar to that of diestrus.
Change in Kiss1 mRNA expression by manipulation of lactation conditions

In each group, Kiss1 mRNA-expressing neurons were observed in both the AVPV and the ARC (Fig. 2A). Compared with non-lactating groups, normal lactating groups displayed significantly lower expression of Kiss1 mRNA in both the AVPV and ARC. The numbers of Kiss1 mRNA-expressing neurons were significantly higher in the 4-h PS and 18-h PS groups compared with normal lactating (non-PS) controls (Fig. 2B) in both AVPV and ARC. The numbers of Kiss1 mRNA-expressing neurons in the AVPV were suppressed by 1-h re-suckling to a level comparable to that in normal lactating rats. In the ARC, the number of Kiss1 mRNA-expressing neurons in the 1-h re-suckling group was at a level intermediate between that of normal lactating and 4-h PS. The numbers of Kiss1 mRNA-expressing neurons in non-lactating groups were significantly higher than normal lactating controls.

Changes in kisspeptin-immunoreactive neurons by manipulation of lactation conditions

Kisspeptin-immunoreactive (kiss-ir) neurons were observed in both the AVPV and ARC (Fig. 3A, and Fig. S1). The change in kiss-ir neurons was similar to that observed for Kiss1 mRNA expression, but less marked. Statistical significance was observed only
between the normal lactating and 18-h RS groups in both the AVPV and the ARC (Fig. 3B).

Afferent input to kisspeptin neurons from the subparafascicular parvocellular nucleus (SPFpc).

Previous studies on lactating animals have identified several neuronal populations or brain nuclei that respond to the suckling stimulus including some brainstem areas such as the peripeduncular nucleus, SPFpc, and caudal area of the periaqueductal gray (Li, et al. 1999; Lonstein and Stern 1999; Marina, et al. 2002; Szabo et al. 2010). In this study, we focused on the SPFpc as a most probable candidate of the relay nucleus of the suckling stimuli to kisspeptin neurons, because the subpopulation of the neurons in the SPFpc has been reported to project fibers to neurons in the ARC (Szabo et al. 2010). To examine the possibility of direct neuronal connection between the SPFpc and kisspeptin neurons, anterograde tracer injection was performed (Fig. 4A). Projection fibers from the SPFpc were seen in both the AVPV (Fig. 4B-E) and the ARC (Fig. 4F-I). In the AVPV, the majority of projection fibers were seen on the ipsilateral side of the injection area, and small amounts of labeled fibers were seen on the contralateral side, whereas the projection fibers in the ARC exclusively resided on the ipsilateral side.
High-magnification photomicrographs showed projection fibers in close apposition to the cell bodies of kisspeptin neurons in both the AVPV (Fig. 4D, E) and ARC (Fig. 4G-I). Close apposition of the BDA-labeled fibers to the cell bodies were seen in all four animals used in the experiment in the ARC, and in three rats in the AVPV.

Change in Kiss1 mRNA expression following continuous administration of prolactin

Serum concentrations of PRL showed significant changes in association with the manipulation of lactating conditions (Fig. 5A). Serum PRL slightly decreased in the 4-h PS group compared with normal lactating controls. However, a significant increase in serum PRL was observed after 1h re-suckling compared with 4-h PS. In non-lactating groups, serum concentrations of PRL were lower than that of normal lactating animals.

Kisspeptin expression in the AVPV and ARC after 1-h continuous PRL administration was assayed using in situ hybridization to examine the possible involvement of changes in serum PRL on re-suppression of Kiss1 mRNA expression (Fig. 5B). Female rats were pup deprived and treated with bromocriptine 18 h prior to PRL infusion to mimic suckling deprivation-induced Kiss1 mRNA up-regulation and to ensure a low concentration of serum PRL. Kiss1 mRNA expression in saline-administered control rats displayed levels comparable to that of 18-h PS rats in the previous experiment.
One-hour administration of PRL significantly suppressed Kiss1 expression in the AVPV, but not in the ARC (Fig. 5B).

**Discussion**

In the present study, we investigated the change in Kiss1 mRNA expression and kisspeptin peptide following modulation of the suckling stimulus, and identified possible mechanisms mediating the suckling stimulus and Kiss1 mRNA expression. Our data provides evidence supporting a significant role of kisspeptin as a modulator of the acute change in LH secretion after modulation of the suckling stimulus reported in previous studies. Our results further indicate that the lactation-induced rapid modulations of kisspeptin are mediated by both direct neuronal input and serum PRL in the AVPV, and by direct neuronal input in the ARC.

Several studies report the reduced expression of kisspeptin under lactating condition using rats (Araujo-Lopes et al. 2014; True et al. 2011; Yamada et al. 2012; Yamada et al. 2007) and mice (Liu et al. 2014). In this study, normal lactating rats showed reduced Kiss1 mRNA levels in the ARC in comparison with that of non-lactating animals, which is consistent with previous studies. Regarding the AVPV, controversial results have been reported in previous studies. Yamada et al. reported almost no detectable basal Kiss1
mRNA in ovariectomized (OVX) lactating rats (Yamada et al. 2012), whereas True et al. reported a decrease in Kiss1 mRNA and an increase in kiss-ir neurons in intact lactating rats (True et al. 2011). In this study, reduced Kiss1 levels in the AVPV of gonadal intact lactating rats was largely consistent with that of True et al (True et al. 2011), indicating that the inconsistency in kisspeptin synthesis in the AVPV is mainly due to the effect of OVX. Although OVX rats are a good model to examine estrogen responsiveness and are widely used in lactating studies, the gonadal intact model reflects the native condition. Our previous study, and several other studies, has reported that kiss-ir cell bodies in the AVPV of non-lactating rats are low or non-detectable (Iijima et al. 2011; Overgaard, et al. 2013). However, in this study, kiss-ir cell bodies were detected in all the groups, even in the normal lactating group in which Kiss1 mRNA was suppressed. Detectable kiss-ir in the cell bodies in the AVPV was also reported by True et al (True et al. 2011). True et al. also showed a lactation-induced decrease in kiss-ir cells in the ARC compared with intact diestrus controls. However, no significant change in kiss-ir between the lactation group and parous non-lactating group was observed in this study. These results indicate the possibility that lactation and birth experience suppresses the kisspeptin peptide release and/or axonal transport of kisspeptin. The numbers of the kiss-ir neurons are relatively smaller than that of Kiss1 mRNA expressing neurons.
observed in the *in situ* hybridization in all the experimental groups. It should be noted that such discrepancies between the *Kiss1* mRNA and the kisspeptin peptide have been reported in several studies (Iijima et al. 2011; Overgaard et al. 2013). These discrepancies could be due to the combination of several reasons including the turnover rate of the molecule, axonal transport of the peptide, and the accumulation in the neuronal cell bodies.

Taking into consideration the reported time-course of LH secretion after a change in suckling stimulus (12–48h) (Maeda et al. 1989), a significant increase in kisspeptin in the 4-h PS group, and the subsequent decrease after 1-h re-suckling in both the AVPV and ARC (Fig. 2) suggests that a rapid change in kisspeptin expression is a direct cause of the change in GnRH/LH secretion. Rapid changes in these models also provide evidence for the rapid turn-over rate of *Kiss1* mRNA. The time-lag between the change in *Kiss1* and the recovery of LH secretion may be due to the time needed for the translation and accumulation of the kisspeptin peptide.

Because the change in kisspeptin after manipulation of the suckling condition is rapid, we first examined the direct neuronal modulation of kisspeptin neurons by the suckling stimulus. Recently, Szabo et al. reported that neuronal projections from the SPFpc, a relay nucleus of the suckling stimulus, innervate into the ARC (Szabo et al. 2010).
However, they did not clarify if the neurons receiving input from the SPFpc were kisspeptin neurons. Our present study provides evidence for a connection between the SPFpc and ARC. Several studies have identified various hormones that modulate the activity of kisspeptin neurons including estradiol, leptin, glucocorticoids and PRL (Araujo-Lopes et al. 2014; Herbison 2008; Smith, et al. 2006a; Takumi, et al. 2012). Electrophysiological studies also have identified several afferent neurotransmitters on kisspeptin, including glutamate, gamma aminobutyric acid (GABA), cocaine- and amphetamine-regulated transcript (CART), and neurokinin B (de Croft, et al. 2013; True, et al. 2013; Zhang, et al. 2013). However, identification of the origin of the afferent projections on kisspeptin neurons are insufficient except for a study by Williams et al. (Williams, et al. 2011) reporting afferent input on AVPV kisspeptin neurons of vasopressin fibers from the suprachiasmatic nucleus. This limited understanding of the origins of afferent inputs on kisspeptin neurons may partially be because of the difficulty in applying conventional retrograde tracing experiments to the dispersed space distribution of kisspeptin neurons, particularly in the ARC. Anterograde tracing in this study discovered a new direct afferent pathway to kisspeptin neurons, from the SPFpc to kisspeptin neurons in both the AVPV and ARC. Brown et al. reported that elimination of sensory inputs from one side of the body by unilateral thelectomy did not affect kiss-ir
cells in both the ipsilateral side and contralateral side of the AVPV (Brown, et al. 2014). Small, but significant contralateral projections from the SPFpc to the AVPV were observed in this study, providing a possible explanation for their results. Although our study discovered the neuronal input to kisspeptin neurons, this may be a fraction of the total afferent pathway to kisspeptin neurons. Further investigation is needed to completely understand the importance of afferent input to kisspeptin neurons.

We next examined the possibility that serum PRL plays a role in rapid modulation of Kiss1 mRNA expression, because accumulating evidence suggests a relationship between the kisspeptin system and PRL secretion. Changes in serum PRL levels via modulation of lactating conditions were largely consistent with the idea that high serum concentrations of PRL are required for continued milk production (Mattheij, et al. 1984). PRL receptor expression in kisspeptin neurons (Kokay, et al. 2011; Li, et al. 2011), and pSTAT5 expression in kisspeptin neurons after intracerebroventricular administration of PRL (Araujo-Lopes et al. 2014) indicates that kisspeptin neurons are responsive to PRL. Several studies report that PRL negatively modulates kisspeptin expression; Sonigo et al. reported reduced expression of kisspeptin in both the AVPV and ARC using chronically PRL-administered hyperprolactinemia model mice (Sonigo, et al. 2012), Araujo-Lopes et al. also reported a reduction in kisspeptin in the ARC following daily injection of the
PRL over 3 days in rats (Araujo-Lopes et al. 2014), and Brown et al. reported reduced expression of Kiss1 in the AVPV of the prolactin injected lactating mice (Brown et al. 2014). Although the difference in PRL levels between 4h PS and non-lactating groups was non-significant, PRL levels showed a rough inverse relationship with Kiss1 expression in the AVPV, which was largely consistent with the idea that PRL has a suppressive effect on Kiss1 expression. Additionally, reduced kisspeptin expression in the AVPV induced by 1 h administration of PRL indicated that kisspeptin responded more rapidly than previously reported.

Regarding Kiss1 expression in the ARC, changes in Kiss1 in the ARC after PS and re-suckling was less marked compared with that in the AVPV, and PRL administration did not have a significant effect on Kiss1 in the ARC. These results apparently contradict previous studies reporting clear changes in Kiss1 using long-term experimental paradigms. One plausible explanation is that the response of Kiss1 in the ARC to extrinsic modulators is slower than that of the AVPV, and consequently the change in Kiss1 in the ARC requires more time to reach statistical significance.

PRL secretion from the pituitary is negatively controlled by tuberoinfundibular dopaminergic (TIDA) neurons, and serum PRL levels remain low in the presence of dopamine from TIDA neurons in non-lactating and virgin animals (Gudelsky 1981).
Suckling stimulus suppresses the expression of tyrosine hydroxylase, a key enzyme of dopamine production, in TIDA neurons, which results in the secretion of PRL from the pituitary (Berghorn, et al. 2001). Recent studies have shown that kisspeptin neurons in the ARC directly project their axons to TIDA neurons (Sawai, et al. 2012), and that kisspeptin suppresses the activity of TIDA neurons (Szawka, et al. 2010). The afferent input from the SPFpc to kisspeptin neurons in the ARC observed in this study indicates a new regulatory mechanism of PRL secretion via a SPFpc-kisspeptin neuron-TIDA neuron feedback pathway. However, the physiological relevance of this pathway remains unclear, and is further complicated by the finding that kisspeptin expression is suppressed in lactating animals.

Because the lactation-induced suppression of the reproductive axis is an important adaptive system for offspring survival, intense research has been conducted to understand the molecular mechanisms of this suppression. Accumulating evidence has confirmed the importance of the kisspeptin system in the lactation-induced suppression of the reproductive axis. We provide here evidence that the rapid modulation of kisspeptin underlies suckling-induced changes in GnRH/LH secretion. The current study provides further morphological evidence of afferent neuronal input to kisspeptin neurons from the SPFpc, a relay nucleus of the suckling stimulus. The rapid change to
the kisspeptin system may be mediated by afferent sensory input via the
SPFpc-kisspeptin neuron pathway, and also partially by serum concentrations of PRL.

**Declaration of interest**

The authors declare that there is no conflict of interest.

**Funding**

This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grants-in-Aid for Scientific Research, Grant Number 15K20062 to SH, 26460323 to H.O. and 26670115 to N.I.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Author contributions**

SH, SA, and HO conceived and designed the experiments. SH and SA performed the experiments and analyzed the data. SH, NI, and HO wrote the paper.

**Acknowledgement**

We are grateful to Takeda pharmaceutical Co. Ltd. for providing a plasmid containing
full-length cDNA of rat Kiss1 for RNA probe synthesis, and providing a monoclonal antibody for kisspeptin.

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Figure legends

Figure 1. Experimental conditions and sampling

Lactating conditions and time of sampling are indicated in the figure. Black bars and hatched bars indicate normal lactation conditions and pup-separated conditions, respectively.

Figure 2. Change in Kiss1 mRNA-expressing neurons following manipulation in lactation conditions.

A Representative images of Kiss1-positive neurons in the AVPV (upper column) and ARC (lower column). Scale bar; 100 μm. 3V; third ventricle. B Quantification of the Kiss1-positive neurons in the AVPV and ARC. The numbers in the bar graphs represent the number of animals in each group. Same alphabetical annotation in B denotes non-significant differences between groups (One-way ANOVA, p<0.05).

Figure 3. Change in kisspeptin-immunoreactive neurons following manipulation in lactation conditions.

A Representative images of kiss-ir neurons in the AVPV (upper column) and ARC (lower column). Insets in the photomicrographs of the ARC show the magnified image
of the cell bodies in the framed area of corresponding images. Scale bar; 100 µm in the AVPV, 50 µm in the ARC. 3V; third ventricle. B Quantification of the kissp-ir neurons in the AVPV and ARC. The numbers in the bar graphs represent the number of animals in each group. Asterisk denotes significant difference between groups (One-way ANOVA, p<0.05).

Figure 4. Simultaneous visualization of kisspeptin neurons and the projections from the SPFpc.

Coronal sections of the brain were double-stained for kisspeptin (brown, DAB) and BDA (black fiber, nickel-cobalt DAB). A Upper panel shows a representative image of the BDA injection site including the SPFpc (black arrow). Lower panel shows schematic illustration of transection of the midbrain containing SPFpc (AP -4.9 mm from Bregma). aq; cerebral aqueduct. B, C Microphotograph of a section containing the AVPV shows kisspeptin-positive neurons and BDA fibers from the SPFpc. 3V; third ventricle. D, E High-magnification images of the framed area in C. Microphotograph of a section containing the ARC is shown in F. G-J High-magnification images of framed area in F. BDA-labeled fibers were seen in close apposition to cell bodies of kisspeptin neurons in both the AVPV (B, C) and ARC (G-J) (black arrow head). The rats used in
the experiment were treated with colchicine to enhance kiss-ir. Scale bars; 500 µm for 
A, 100 µm for B, C, and F, 10 µm for D, E, G, H, and I.

Figure 5. Kiss1 mRNA expressions after 1h continuous administration of prolactin.

Serum concentrations of PRL under modified lactating conditions are shown in A. Same 
alphabetic annotation denotes non-significant difference between groups (One-way 
ANOVA, p<0.05). Representative images of Kiss1 neurons in the AVPV and ARC after 
PRL administration and comparison of the number of Kiss1-positive neurons between 
PRL treated and control rats are shown in B. The numbers in the bar graphs represent 
the number of animals in each group. Scale bar; 100µm. Asterisk denotes significant 
differences between groups (Students’ t-test, p<0.05).
Figure 1

Birth
Day0

Milk-feeding

Pup Separation (PS)

Sampling
Day8 11:00-14:00

normal lactating

4h PS

4h PS + re-suckling

18h PS

non-lactating

49x28mm (300 x 300 DPI)
Figure 2

A. normal lactating  PS 4h  PS 4h re-suckling 1h  PS 18h  non-lactating

AVPV

ARC

B. AVPV

ARC

Mean number of
Kisspeptin cells/section

147x121mm (300 x 300 DPI)
Figure 3

A  
- normal lactating
- PS 4h
- PS 4h re-suckling 1h
- PS 18h
- non-lactating

AVPV

ARC

B

AVPV  
- Bar chart showing mean number to kisspeptin cells per section
  - Normal lactating
  - PS 4h
  - PS 4h re-suckling 1h
  - PS 18h
  - Non-lactating

ARC

147x121mm (300 x 300 DPI)