

Central somatostatin-somatostatin receptor 2 signaling mediates lactational suppression of luteinizing hormone release via the inhibition of glutamatergic interneurons during late lactation in rats

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Abstract. Reproductive function is suppressed during lactation owing to the suckling-induced suppression of the kisspeptin gene (*Kiss1*) expression in the arcuate nucleus (ARC) and subsequent suppression of luteinizing hormone (LH) release. Our previous study revealed that somatostatin (SST) neurons mediate suckling-induced suppression of LH release via SST receptor 2 (SSTR2) in ovariectomized lactating rats during early lactation. This study examined whether central SST-SSTR2 signaling mediates the inhibition of ARC *Kiss1* expression and LH release in lactating rats during late lactation and whether the inhibition of glutamatergic neurons, stimulators of LH release, is involved in the suppression of LH release mediated by central SST-SSTR2 signaling in lactating rats. A central injection of the SSTR2 antagonist CYN154806 (CYN) significantly increased ARC *Kiss1* expression in lactating rats on day 16 of lactation. Dual *in situ* hybridization revealed that few ARC *Kiss1*-positive cells co-expressed *Sstr2*, and some of the ARC *Slc17a6* (a glutamatergic neuronal marker)-positive cells co-expressed *Sstr2*. Furthermore, almost all ARC *Kiss1*-positive cells co-expressed *Grin1*, a subunit of N-methyl-D-aspartate (NMDA) receptors. The numbers of *Slc17a6/Sstr2* double-labeled and *Slc17a6* single-labeled cells were significantly lower in lactating dams than in non-lactating rats whose pups had been removed after parturition. A central injection of an NMDA antagonist reversed the CYN-induced increase in LH release in lactating rats. Overall, these results suggest that central SST-SSTR2 signaling, at least partly, mediates the suppression of ARC *Kiss1* expression and LH release by inhibiting ARC glutamatergic interneurons in lactating rats.

Key words: Glutamate, Kisspeptin, Lactation, Luteinizing hormone, Somatostatin

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Ovarian functions, such as follicular development and ovulation, are severely suppressed during lactation in species such as rodents, ruminants, and primates, including humans, resulting in lactational anestrus in these species [1–3]. This suppression is assumed to be primarily due to the suckling-induced inhibition of pulsatile release of gonadotropin-releasing hormone (GnRH) and the subsequent inhibition of gonadotropin release. Luteinizing hormone (LH) pulses were strongly suppressed in lactating rats, and LH pulses were restored several hours after the removal of pups from lactating rats [4–7].

Previous studies have revealed that the suppression of pulsatile GnRH/LH release in lactating rats is largely due to a profound inhibition of kisspeptin and kisspeptin gene (*Kiss1*) expression in the hypothalamic arcuate nucleus (ARC) [8, 9]. The ARC kisspeptin neurons co-express neurokinin B (*Tac3*) and dynorphin A (*Pdyn*) genes, which are referred to as KNDy neurons [10–12]. The KNDy neurons are considered to be the GnRH/gonadotropin pulse generator in mammals, including rodents and ruminants [12–15]. Our previous study revealed that among the KNDy genes, *Kiss1* gene expression, but not *Tac3* or *Pdyn* gene expression, was strongly suppressed in the

ARC of ovariectomized (OVX) lactating rats during early lactation [16]. Furthermore, the study suggested that somatostatin (SST) and SST receptor 2 (SSTR2, an SST receptor) signaling at least partially mediates the suppression of pulsatile LH secretion during early lactation as the central administration of SSTR2 antagonist increased LH release in OVX lactating rats. Additionally, the expression of the SST gene (*Sst*) in the thalamus and SSTR2 gene (*Sstr2*) in the ARC was significantly increased in lactating rats compared to that in non-lactating controls [16]. In addition, we suggested that SST might indirectly act on ARC kisspeptin neurons via interneuron(s) in the hypothalamus, as histological examination revealed low *Sstr2* expression in the ARC kisspeptin neurons (< 1%) in lactating rats [16]. Glutamatergic neurons are potential interneurons as glutamate is a major stimulatory neurotransmitter for GnRH/LH secretion and acts by stimulating kisspeptin neurons. Glutamatergic neurons are abundantly located in the ARC in female rats [17–20]. N-methyl-D-aspartate (NMDA), an ionotropic glutamatergic receptor agonist, immediately stimulates LH secretion in wild-type female rats but not in *Kiss1* knockout rats [21]. Furthermore, our previous studies have revealed that the expression of NR1 gene (*Grin1*), a subunit of the NMDA receptor, can be observed in most ARC kisspeptin neurons in female rats [22], and glutamate treatment largely enhanced *in vitro* GnRH release from the ARC-median eminence tissue taken from female rats [23]. Notably, previous studies have indicated the suppressive effect of SST-SSTR2 signaling on glutamate release from glutamatergic neurons in the cerebral cortex and retina of mice [24, 25] and in the hippocampus and basal forebrain of rats [26, 27]. These results indicate that glutamatergic interneurons may mediate suckling-induced suppression of LH release by serving as an action

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site of SST-SSTR2 signaling during lactation.

The present study examined whether SST-SSTR2 signaling is involved in the suckling-induced suppression of ARC *Kiss1* expression and LH secretion and whether the ARC glutamatergic neurons act as interneurons in mediating the suckling-induced suppression of LH release by SST-SSTR2 signaling in rats during late lactation. To address these issues, we examined the effect of central administration of an SSTR2 antagonist on ARC *Kiss1* expression in ovary-intact lactating rats on day 16 of lactation. Furthermore, we investigated the expression of *Sstr2* or *Grin1* in ARC *Kiss1*-expressing neurons using double *in situ* hybridization (ISH) in estradiol-17 β (E2)-treated OVX non-lactating rats. We also investigated whether the suckling stimulus reduces the expression of *Slc17a6*, a glutamatergic neuronal marker gene, by comparing the expression of *Slc17a6* between lactating and non-lactating rats. We also investigated the co-expression of *Sstr2* and *Slc17a6* by double ISH in the ARC of lactating and non-lactating rats. Finally, we investigated whether a central injection of an NMDA antagonist reverses the SSTR2 antagonist-induced increase in LH release in lactating rats.

Materials and Methods

Animals and experimental design

Wistar-Imamichi female rats (Institute for Animal Reproduction, Kasumigaura, Japan) were housed in a controlled environment (14 h light/10 h dark cycle; temperature at 22 \pm 3°C) with free access to food and water. Female rats with at least two consecutive estrous cycles were mated with male rats. Pregnant female rats were housed individually until the day of parturition, and the day of parturition was designated as day 0 of lactation. The litter size in the lactating group was adjusted to eight on day 1. Some female dams were deprived of their litters on day 1, were OVX, and then subcutaneously implanted with a Silastic tubing (inner diameter 1.5 mm; outer diameter 3.0 mm; length 25 mm; Dow Corning, Midland, MI, USA) containing E2 (Sigma Aldrich, St Louis, MO, USA) dissolved in peanut oil (Sigma Aldrich) at 20 μ g/ml to mimic the plasma E2 levels in intact lactating rats [9, 28, 29] to act as OVX + low E2 non-lactating rats. OVX and low E2 treatments were administered to avoid steroidal fluctuations associated with estrous cycles after pup removal. This treatment has been confirmed to suppress LH release during late lactation, as shown in ovary-intact lactating rats [9]. The low E2 treatment did not exhibit an inhibitory effect on ARC *Kiss1* expression in virgin OVX female rats and non-lactating OVX rats 16 days after parturition [9, 30]. The ovary-intact lactating dams were used to examine the effect of central SSTR2 antagonism on the expression of *Kiss1* in the ARC during the late lactation period (day 16 of lactation). Some lactating and non-lactating rats were used to compare *Sstr2* and *Slc17a6* expression in the ARC between lactating and non-lactating conditions. Furthermore, some lactating rats were used to investigate the effect of central glutamatergic antagonism on LH release recovered by the SSTR2 antagonist. Expression of *Sstr2* or *Grin1* in ARC *Kiss1*-expressing cells was determined in non-lactating rats. The non-lactating rats were used as ARC *Kiss1* expression is largely inhibited in lactating dams [9]. Thus, some ARC *Kiss1*-expressing cells could not be identified in the lactating rats. If not otherwise specified, the surgical procedures were performed under anesthesia with an intraperitoneal injection of ketamine (27 mg/kg; Fujita, Tokyo, Japan)/xylazine (5.3 mg/kg; Bayer AG, Leverkusen, Germany) mixture followed by inhalation of 1% to 2% isoflurane (Pfizer Japan, Tokyo, Japan). The present study was approved by the Committee on Animal Experiments of

the Graduate School of Bioagricultural Sciences, Nagoya University.

Administration of central SSTR2 antagonist with/without NMDA antagonist and blood sampling

Lactating or non-lactating rats were stereotaxically implanted with a stainless-steel guide cannula (22 gauge; P1 Technologies, Roanoke, VA, USA) for intracerebroventricular (icv) injection into the third ventricle (3V) on day 7 of lactation to investigate the effects of SSTR2 antagonist on ARC *Kiss1* expression or LH pulses. The brain coordinates for the 3V injection were 0.8 mm posterior and 7.5 mm ventral to the bregma according to the rat brain atlas [31].

CYN154806 (CYN, Sigma Aldrich), an SSTR2-specific antagonist that binds to SSTR2 with high affinity (pIC₅₀ 8.6) and to other SST receptors with low affinity (pIC₅₀ 5.4–6.5) [32], was dissolved in ultrapure water (10 nmol/2 μ l) and MK801 (Sigma Aldrich), an NMDA receptor antagonist, was dissolved in artificial cerebrospinal fluid (CSF) (100 nmol/3 μ l). Free-moving conscious lactating rats were administered CYN (n = 4) to the 3V at a flow rate of 1 μ l/min for 2 min on day 16 of lactation, using a microsyringe pump (EICOM, Kyoto, Japan) immediately after the first blood collection. Some lactating rats were administered MK801 (n = 4) to the 3V at a flow rate of 1 μ l/min for 3 min followed by the 3V CYN administration. Control lactating rats (n = 4) were administered vehicle at the 3V. The CYN dose was selected according to our previous study, which revealed that the same dose of CYN significantly increased LH release in OVX lactating mother rats during early lactation (day 8 of lactation) [16]. The dose of MK801 was chosen according to a previous study, which showed that an icv injection of the same dose of MK801 blocked *fos* expression induced by dehydration in the median preoptic nucleus, supraoptic nucleus, and paraventricular nucleus in male rats [33]. Blood samples (150 μ l) were collected every 6 min for 3 h through a silicone cannula (Shin-Etsu Polymer, Tokyo, Japan) that had been inserted into the right atrium via the jugular vein on the day before blood sampling. An equivalent volume of rat red blood cells, taken from donor rats and diluted with heparinized saline, was replaced through the atrial cannula after each blood collection. Plasma samples (50 μ l) were obtained by immediate centrifugation and stored at –20°C until assayed for LH. At the end of blood sampling, the placement of the icv cannula was verified by visual inspection after the injection of the same amount of 3% brilliant blue solution using a microsyringe pump.

LH assays

Plasma LH concentrations were measured by a double-antibody radioimmunoassay (RIA) using a rat LH RIA kit provided by the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, USA) and were expressed in terms of the NIDDK rat LH-RP-3. The lowest detectable level was 7.8 pg/tube for 50 μ l plasma samples. Intra- and inter-assay coefficients of variation were 6.7% and 10.3%, respectively, at the level of 0.78 ng/ml.

Preparation of complementary RNA (cRNA) probes for ISH

Digoxigenin (DIG)-labeled antisense cRNA probes were designed for *Kiss1* (GenBank accession number AY196983, nucleotide 33–348), *Sstr2* (NM_019348, nucleotide 176–1092), *Grin1* (NM_001270602, nucleotide 768–1678 and 1678–2609), and fluorescein isothiocyanate (FITC)-labeled antisense cRNA probes were designed for *Slc17a6* (NM_053427.1, nucleotide 935–2053) and *Kiss1*. These probes were synthesized via *in vitro* transcription using complementary DNA (cDNA) obtained from the rat whole hypothalamus using DIG- or FITC-labeling mix (Roche Diagnostics, Basel, Switzerland), with

the appropriate polymerase (T7 or T3) according to the direction of the cDNA insertion. The identification of cDNA insertions was confirmed using Sanger sequencing.

Brain sampling and single- and double-ISH for analysis of Kiss1, Sstr2, Grin1, and Slc17a6 expression in the ARC of lactating and non-lactating rats

Brain samples were collected from lactating and non-lactating rats 16 days after parturition. Some free-moving conscious lactating rats on day 16 of lactation were administered CYN or vehicle ($n = 3-5$) into the 3V as described above. Some non-lactating rats ($n = 3$) on postpartum day 16 were administered the vehicle at the 3V. One hour after injection, the animals were deeply anesthetized with sodium pentobarbital (40 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) and immediately perfused with 0.05 M phosphate-buffered saline (PBS) and subsequently with 4% paraformaldehyde (PFA) in 0.05 M phosphate buffer (PB). The brains were removed from the skulls and post-fixed with 4% PFA overnight at 4°C and retained in 30% sucrose in 0.05 M PB at 4°C until they sank under RNase-free conditions for 4 days. Serial hypothalamic coronal sections (50 μm in thickness) through the ARC, prepared using a cryostat (CM1800, Leica Biosystems, Wetzlar, Germany), were subjected to single ISH for *Kiss1* ($n = 3-5$) or double ISH for *Sstr2* and *Slc17a6* ($n = 3$). Brain sections obtained from non-lactating rats ($n = 3$) were subjected to double ISH for *Kiss1* and *Sstr2*, or *Kiss1* and *Grin1*.

Free-floating single ISH for *Kiss1* was conducted as previously described [34, 35]. Briefly, every fourth rat brain section was used for analyzing the ARC (13 sections, 1.72 to 4.36 mm posterior to the bregma) according to the rat brain atlas [31]. The sections were washed with 0.05 M PBS, treated with 1 $\mu\text{g}/\text{ml}$ protease K for 15 min at 37°C, and incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were hybridized with 1.0 $\mu\text{g}/\mu\text{l}$ DIG-labeled antisense cRNA probes overnight at 60°C. After hybridization, the sections were washed twice with $2 \times$ saline-sodium citrate (SSC)-50% formamide for 15 min at 60°C, treated with 20 $\mu\text{g}/\text{ml}$ RNase A for 30 min at 37°C, and steeped in $2 \times$ SSC, 0.5 \times SSC, and DIG-1 buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% Tween-20) for 15 min twice. The sections were soaked in 1.5% blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany) in DIG-1 buffer for 1 h at 37°C and incubated with alkaline phosphatase-conjugated anti-DIG Fab fragment (1:1000; #11093274910, Roche Diagnostics; RRID: AB_2734716) for 2 h at 37°C. Subsequently, the sections were washed twice with DIG-1 buffer and then with DIG-3 buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl_2). The hybridized and immuno-labeled probes were visualized with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate in DIG-3 buffer. The visualization reaction was terminated by a reaction stop solution (10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid, pH 8.0). The sections were observed under an Olympus BX53 light microscope, and the number of *Kiss1*-expressing cells throughout the ARC was bilaterally counted thrice in each section and then averaged.

Free-floating double labeling ISH was conducted, as described previously [16, 36], for the following combinations: *Kiss1* and *Sstr2*, *Kiss1* and *Grin1*, and *Slc17a6* and *Sstr2*. Briefly, every fourth rat brain section containing the ARC was hybridized with 1.0 $\mu\text{g}/\mu\text{l}$ FITC- and DIG-labeled antisense cRNA probes overnight at 60°C. The hybridized FITC-labeled probe was detected using peroxidase-conjugated anti-FITC Fab fragment (1:1000, #11426346910, Roche Diagnostics; RRID: AB_840257) and the TSA Plus FITC Kit (Perkin Elmer, Waltham, MA, USA), according to the manufacturer's instructions.

After inactivation of the peroxidase by incubating the brain sections in 0.1 N hydrochloric acid for 30 min, the DIG-labeled probe was detected using peroxidase-conjugated anti-DIG Fab fragment (1:500, #11207733910, Roche Diagnostics; RRID: AB_514500), TSA Plus Biotin Kit (Perkin Elmer), and DyLight594-conjugated streptavidin (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescent images were captured and investigated under a fluorescence microscope with ApoTome optical sectioning (Carl Zeiss, Oberkochen, Germany). The numbers of *Kiss1*-, *Sstr2*-, *Grin1*-, and *Slc17a6*-expressing cells throughout the ARC were unilaterally counted thrice in each section and then averaged.

Statistical analysis

LH pulses were identified by the PULSAR computer program [37-39], and the mean LH concentrations and frequency and amplitude of LH pulses for a 3 h sampling period were calculated for each individual and then for the group. Statistical differences in the LH pulse parameters (mean LH concentrations and frequency of LH pulses) between groups, and the number of *Kiss1*-, *Slc17a6*-, *Slc17a6*/*Sstr2*-, and *Sstr2*-expressing cells in the ARC between groups were determined by one-way ANOVA followed by the Bonferroni test. Statistical differences in the amplitude of LH pulses between vehicle- and CYN-treated groups were determined using the Student's *t*-test. All tests were conducted using R statistical software (release 3.5.1).

Results

Central SSTR2 antagonism increased the ARC Kiss1 expression in lactating rats

Figure 1a shows the photomicrographs of *Kiss1*-expressing cells in the ARC of representative non-lactating rats treated with vehicle at the 3V, and those of lactating rats treated with CYN or vehicle at the 3V. *Kiss1* signals were strongly and abundantly found in the ARC of non-lactating rats. *Kiss1* signals were also found in the ARC of both vehicle- and CYN-treated lactating dams, and were weaker than those in non-lactating rats. The number of *Kiss1*-expressing cells in the ARC is shown in Fig. 1b. One-way ANOVA revealed a significant difference in the number of ARC *Kiss1*-expressing cells between the groups [$F(2,10) = 32.797$, $P < 0.001$]. In particular, the Bonferroni test revealed that the number of ARC *Kiss1*-expressing cells in vehicle-treated ($P < 0.001$) or CYN-treated ($P < 0.001$) lactating rats was significantly lower than that of vehicle-treated non-lactating rats ($n = 3-5$, Fig. 1b). Importantly, the number of ARC *Kiss1*-expressing cells in CYN-treated lactating rats was significantly higher than that in vehicle-treated lactating rats ($P = 0.032$, Fig. 1b).

Determination of Sstr2 or Grin1 expression in the ARC Kiss1-positive cells in female rats

Figure 2a shows the representative photomicrographs of *Sstr2* and *Kiss1* signals determined by a double ISH in the ARC of non-lactating rats 16 days after parturition. Several *Sstr2*- or *Kiss1*-positive cells were found in the ARC of the animals. Quantitative analysis revealed that 5.6% of *Kiss1*-expressing cells (44.4 ± 15.6 out of 772.8 ± 44.3 cells, $n = 3$) were dual-labeled with *Sstr2* signals in the ARC (Fig. 2b). Figure 2c shows the representative photomicrographs of *Grin1*- and *Kiss1*-positive cells in the ARC of a non-lactating animal. Quantitative analysis revealed that a majority (91.2%, 799.7 ± 37.4 out of 876.0 ± 17.3 cells, $n = 3$) of *Kiss1*-expressing cells showed *Grin1* signals in the ARC of the animals (Fig. 2d).

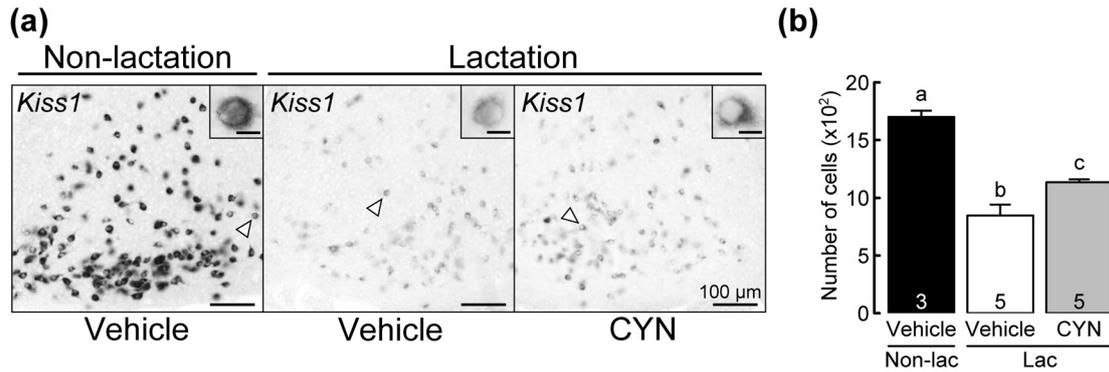


Fig. 1. Effects of central administration of somatostatin receptor 2 (SSTR2) antagonist (CYN) on *Kiss1* mRNA expression in the arcuate nucleus (ARC) of lactating rats. (a) Representative photomicrographs showing *Kiss1* mRNA expression in the ARC of non-lactating rats injected with vehicle in the third ventricle (3V) and lactating rats injected with vehicle or CYN in the 3V 1 h before brain sampling. Insets indicate *Kiss1*-expressing cells at higher magnification. Scale bars in the insets represent 10 μm . (b) Number of *Kiss1*-expressing cells in the ARC of vehicle-treated non-lactating rats (closed column), vehicle-treated lactating rats (open column), and CYN-treated lactating rats (shaded column). Values are the means \pm SEM. Numbers in each column indicate the number of animals used. Values marked with different letters are significantly different ($P < 0.05$, one-way ANOVA followed by Bonferroni test).

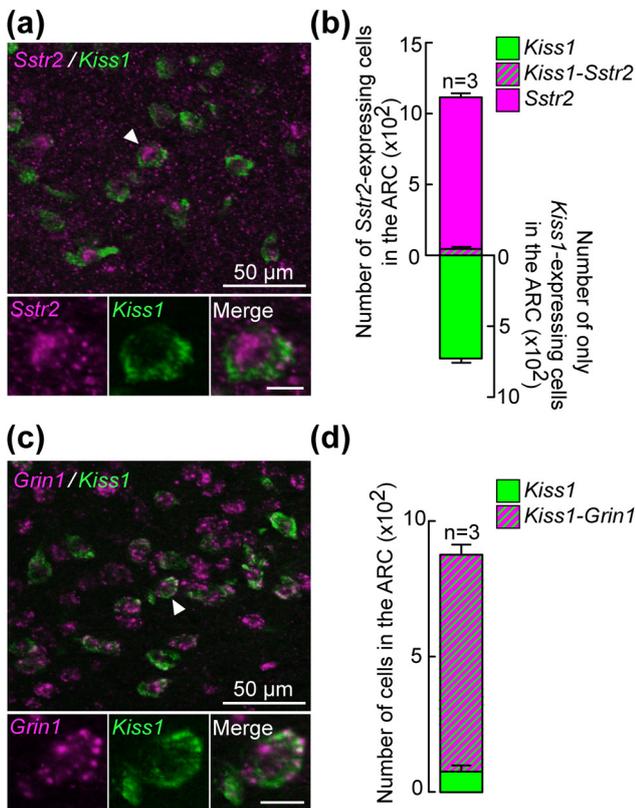


Fig. 2. Co-expression of *Sstr2* (SSTR2 gene) or *Grin1*, an N-methyl-D-aspartate (NMDA) receptor marker gene, in the ARC *Kiss1*-expressing cells of non-lactating rats. (a) Representative photomicrographs showing *Sstr2*- (magenta) and *Kiss1*- (green) expressing cells in the ARC of non-lactating rats. A dual-labeled cell marked with an arrowhead is shown as magnified images showing *Sstr2*- and *Kiss1*-positive and merged cells. Scale bars in the magnified images represent 10 μm . (b) Number of cells expressing *Sstr2* alone (magenta), both *Sstr2* and *Kiss1* (magenta/green), or *Kiss1* alone (green) in the ARC were quantified and shown in a stacked bar graph. Values are the means \pm SEM. (c) Representative photomicrographs showing *Grin1*- (magenta) and *Kiss1*- (green) expressing cells in the ARC of non-lactating rats. A dual-labeled cell marked with an arrowhead is shown as a magnified image showing *Grin1*- and *Kiss1*-positive and merged cells. (d) Number of cells expressing both *Grin1* and *Kiss1* (magenta/green) or *Kiss1* alone (green) in the ARC were quantified and are shown in a stacked bar graph. Values are the means \pm SEM.

Decrease in the number of *Slc17a6*-expressing cells and of *Slc17a6*- and *Sstr2*-co-expressing cells in the ARC of lactating rats

Figure 3a shows the representative photomicrographs of *Sstr2* and *Slc17a6* signals in the ARC of lactating rats treated with 3V injection of CYN or vehicle and non-lactating rats treated with 3V injection of vehicle. Many *Slc17a6*-expressing cells were found in the ARC of non-lactating rats, whereas fewer *Slc17a6*-expressing cells were found in the ARC of lactating groups. *Sstr2*-positive cells were also found in the ARC in all the groups. One-way ANOVA revealed a significant difference in the number of *Slc17a6*-positive cells between the groups [$F(2,6) = 12.33$, $P = 0.007$]. In particular, the Bonferroni test revealed that the number of *Slc17a6*-positive cells in vehicle-treated ($P = 0.013$) or CYN-treated ($P = 0.018$) lactating rats was significantly lower than that of non-lactating rats (Fig. 3b). No significant difference was found in the number of *Slc17a6*-positive cells between the lactating groups with and without CYN treatment.

Sstr2 signals were found in a part of *Slc17a6*-positive cells in the ARC of non-lactating rats (12.9%, 294.8 \pm 18.5 out of 2312.2 \pm 201.0 *Slc17a6*-expressing cells, $n = 3$), vehicle-treated lactating rats (11.9%, 137.7 \pm 35.5 out of 1137.8 \pm 253.1 *Slc17a6*-expressing cells, $n = 3$), and CYN-treated lactating rats (10.5%, 128.6 \pm 20.9 out of 1221.2 \pm 4.8 *Slc17a6*-expressing cells, $n = 3$). One-way ANOVA revealed a significant difference in the number of *Sstr2*-expressing *Slc17a6*-positive cells [$F(2,6) = 12.858$, $P = 0.007$] between the groups. In particular, the Bonferroni test revealed that the number of *Sstr2*-expressing *Slc17a6*-positive cells in vehicle-treated ($P = 0.016$) or CYN-treated ($P = 0.012$) lactating rats was significantly lower than that of non-lactating rats ($n = 3$, Fig. 3b). No significant difference was found in the number of *Sstr2*-expressing cells between the groups.

Central glutamatergic antagonism blocked the increase in LH release induced by SSTR2 antagonist in lactating rats

Figure 4a shows the LH release profile in lactating rats bearing a 3V injection of vehicle or CYN with or without co-administration of glutamatergic NMDA receptor antagonist (MK801) on day 16 of lactation. LH release was inhibited in vehicle-injected control lactating dams and could be detected in lactating dams treated with 3V CYN. In contrast, LH release was inhibited in CYN-treated

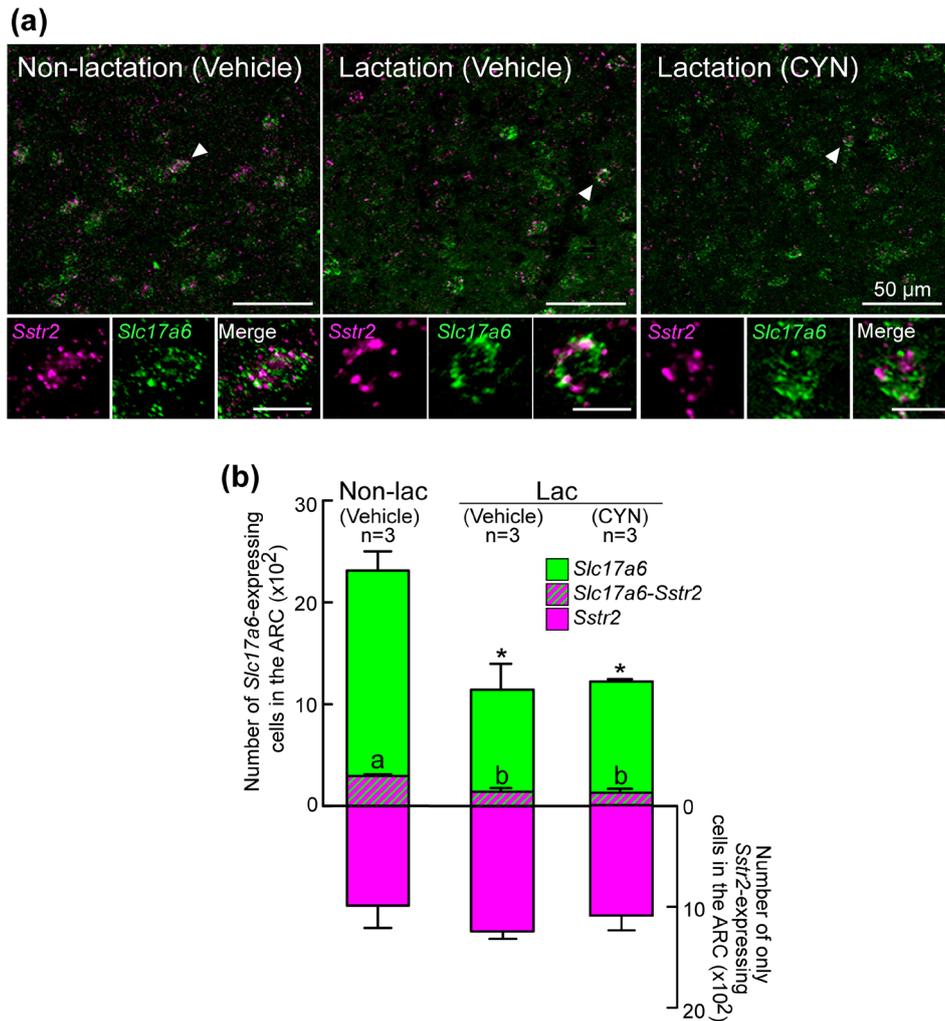


Fig. 3. *Sstr2* (SSTR2 gene) and *Slc17a6* (a glutamatergic neuronal marker gene) expression in the ARC of non-lactating and lactating rats. (a) Representative photomicrographs showing *Sstr2*- (magenta) and *Slc17a6*- (green) expressing cells in the ARC of vehicle-treated non-lactating rats, and vehicle- or CYN-treated lactating rats. A dual-labeled cell marked with arrowheads was shown as magnified images showing *Sstr2*- and *Slc17a6*-positive and merged cells. Scale bars in the magnified images represent 10 μ m. (b) Number of cells expressing *Sstr2* alone (magenta), both *Sstr2* and *Slc17a6* (magenta/green), or *Slc17a6* alone (green) in the ARC of each group were quantified and are shown in a stacked bar graph. Values are shown as the means \pm SEM. Values marked with an asterisk are significantly different ($P < 0.05$, one-way ANOVA followed by Bonferroni test) in the number of *Slc17a6*-expressing cells in the ARC from that in the non-lactating group. Values marked with different letters are significantly different ($P < 0.05$, one-way ANOVA followed by Bonferroni test) from each other in the number of the ARC cells co-expressing *Sstr2* and *Slc17a6*.

lactating rats when MK801 was co-administered into the 3V. One-way ANOVA revealed a significant difference in mean LH concentrations [$F(2, 9) = 88.672, P < 0.001$] between the groups. In particular, the Bonferroni test revealed that the mean LH concentrations in lactating rats treated with CYN were significantly higher than those in vehicle-treated lactating rats ($P < 0.001$), and that the mean LH concentrations in lactating rats treated with both CYN and MK801 were significantly lower than those in the vehicle ($P < 0.001$) or CYN-treated ($P = 0.021$) lactating rats (Fig. 4b). LH pulse frequency tended to increase in CYN-treated animals and decrease in CYN/MK801-treated lactating dams compared to that in vehicle-treated controls. However, significant differences were not observed between groups (Fig. 4c). LH pulse amplitude in lactating animals treated with CYN was significantly higher than that in vehicle-treated controls ($P = 0.005$, Student's *t*-test). Additionally, statistical analysis between the three groups was not applicable for the amplitude of LH pulses as LH pulses were found in two out of four CYN- and MK801-treated lactating dams (Fig. 4d).

Discussion

The present study demonstrates that SST-SSTR2 signaling, at least partly, mediates the suckling-induced suppression of LH release by inhibiting kisspeptin neurons in lactating rats as a central administration of SSTR2 antagonist significantly increased plasma LH levels and *Kiss1* expression in the ARC of rats during late lactation. The present study also suggests that the ARC glutamatergic neurons mediate the suckling-induced suppression of LH release by SST-SSTR2 signaling in lactating rats as central administration of an NMDA antagonist reversed the SSTR2 antagonist-induced increase in LH release in lactating dams. Furthermore, the present study revealed that a part of the ARC *Slc17a6*-positive glutamatergic neurons co-expressed *Sstr2*, most of the ARC *Kiss1*-positive cells co-expressed *Grin1* (NMDA receptor marker gene), and the number of *Slc17a6*-expressing cells decreased in the ARC of lactating rats as compared to that in non-lactating rats. Taken together, these results suggest that the suckling stimulus suppresses ARC *Kiss1* expression and consequent

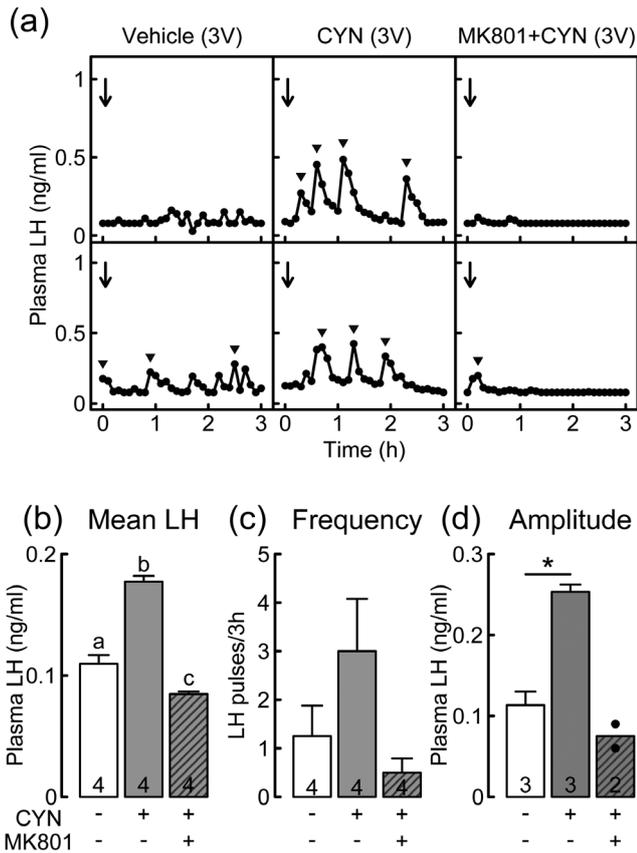


Fig. 4. Effects of the 3V injection of SSTR2 antagonist (CYN) with or without co-administration of glutamatergic NMDA receptor antagonist (MK801) on LH secretion in lactating rats on day 16 of lactation. (a) Profile of plasma LH levels in lactating rats treated with vehicle or CYN in combination with vehicle (CSF) or MK801 immediately after the onset of blood sampling (arrows). Arrowheads indicate the peaks of LH pulses detected by the PULSAR computer program. Mean LH concentrations (b) and the frequency (c) and amplitude of LH pulses (d) of lactating rats in each group. Open, shaded, and hatched bars show values (means \pm SEM) in vehicle-, CYN-, and CYN/MK801-treated lactating rats, respectively. Numbers in each column indicate the number of animals used. Values marked with different letters are significantly different ($P < 0.05$, one-way ANOVA followed by Bonferroni test) from each other. The amplitude of LH pulses between vehicle- and CYN-treated lactating rats is significantly different (* $P = 0.005$, Student's *t*-test).

LH release through, at least partly, inhibitory SST-SSTR2 signaling and suppression of the ARC glutamatergic neurons in lactating rats.

Our previous study revealed that in early lactating OVX rats, central administration of an SSTR2 antagonist significantly restored LH pulses [16]. This study revealed the involvement of SST-SSTR2 signaling in lactational LH suppression during the late lactation period. These findings suggest that SST-SSTR2 signaling is involved in LH suppression throughout the lactation period in rats. The involvement of SST-SSTR2 signaling in suppressing LH release during late lactation in rats is consistent with a previous study, which showed that central SST signaling could be involved in the estradiol negative feedback effects on LH release in ewes [40]. Taken together, these findings suggest that SST-SSTR2 signaling mediates suckling stimulus-induced inhibition of ARC *Kiss1* expression, resulting in the suppression of GnRH/LH release during the entire lactation period in rats.

Glutamatergic neurons stimulate GnRH/LH secretion by activating

kisspeptin neurons [21]. The present study revealed that SSTR2 expression in ARC kisspeptin neurons was extremely limited in female rats, suggesting that SST might indirectly inhibit ARC *Kiss1* expression and consequent GnRH/LH release by affecting stimulatory interneurons for kisspeptin neuronal activity. As expected, the present study suggests that the inhibitory SST-SSTR2 signal, at least partly, affects ARC glutamatergic neurons. This is because the current central NMDA receptor antagonism reversed the increase in LH release induced by the SSTR2 antagonist in lactating dams. Furthermore, *Sstr2* was co-expressed in some ARC glutamatergic neurons, and *Grin1*, a subunit of the NMDA receptor, was evident in most ARC kisspeptin neurons in female rats. Notably, the number of ARC *Slc17a6*-positive glutamatergic cells in lactating dams was significantly lower than that in non-lactating controls, regardless of the SSTR2 antagonist treatment. This suggests that the suckling stimulus reduced the ARC glutamatergic neurons via the SSTR2-independent pathway. Furthermore, this study revealed that numerous non-glutamatergic *Sstr2*-expressing cells were abundantly located in the ARC of lactating dams. These findings imply that interneurons other than glutamatergic neurons may also be involved in the SST-induced inhibition of LH release in late lactating rats. Further studies are required to address these issues.

Notably, the current central administration of the SSTR2 antagonist significantly increased the mean LH levels but failed to affect the LH pulse frequency. These results suggest that SST-SSTR2 signaling may not be involved in the direct inhibition of GnRH pulse generator activity. This notion is supported by the current result that *Sstr2* expression is limited in ARC kisspeptin neurons, namely KNDy neurons, which are responsible for GnRH pulse generation [13]. Furthermore, it is also possible that the current SSTR2 antagonist may directly affect GnRH neurons, as SSTR2 is highly expressed in GnRH neurons and that SST-immunoreactive fibers are closely localized in GnRH neuronal fibers in the organum vasculosum of the lamina terminalis in female rats [41]. Additionally, SST-immunoreactive fibers are appositions with 50–60% of GnRH neurons in male and female mice [42].

In this study, the number of ARC *Sstr2*-expressing cells was comparable between lactating and non-lactating rats during the late lactation period. Our previous study revealed that the number of ARC *Sstr2*-expressing cells in early lactating rats was comparable to that in non-lactating control rats; while *Sstr2* mRNA levels in the ARC-ME region in lactating rats were significantly higher than those in non-lactating rats [16]. These results imply that *Sstr2* mRNA levels per cell would be increased by suckling stimulus in both early and late lactation periods.

Our previous study revealed that the suckling stimulus increased *Sst* expression in the posterior intralaminar complex of the thalamus (PIL) and *Sstr2* expression in the ARC, in which the *Sstr2* expression was higher than other SST receptor mRNAs in rats during early lactation [16]. Thus, as shown in Fig. 5, we envisioned that the suckling stimulus may also activate the PIL SST neurons during late lactation. The resultant direct (on kisspeptin neurons) and indirect (on glutamatergic neurons) SST-SSTR2 signaling may inhibit *Kiss1* expression and subsequent GnRH/LH release during late lactation. Interestingly, Dufourny *et al.* demonstrated that most ARC kisspeptin neurons showed SST appositions in both male and female rats and that one-third of kisspeptin neurons expressed SST receptor 1 (SSTR1)-immunoreactivity in male rats [43]. Thus, SST may directly inhibit ARC kisspeptin neurons via SSTR1 in female rats during lactation. Further studies are needed to understand the roles of SST-SSTR1 signaling in lactational anestrus.

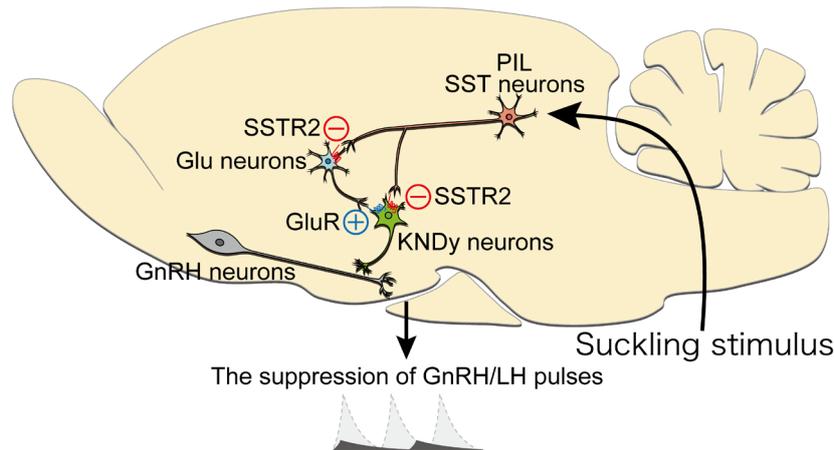


Fig. 5. Schematic showing the possible brain mechanism underlying suckling stimulus-induced inhibition of ARC *Kiss1* expression and consequent GnRH/LH pulses in lactating rats based on the present and previous studies [16]. The suckling stimulus seems to activate the PIL SST neurons, and the resultant inhibitory SSTR2 signaling may result in the inhibition of *Kiss1* expression in KNDy neurons and subsequent GnRH/LH pulses via direct (on kisspeptin neurons) and indirect (on stimulatory glutamatergic neurons) pathways. Glu, Glutamatergic; GluR, Glutamatergic receptor.

In conclusion, the present study demonstrates that SST-SSTR2 signaling, at least partly, mediates suckling-induced suppression of GnRH/LH release and ARC *Kiss1* expression via inhibition of hypothalamic glutamatergic neurons during the late lactating period in rats.

Conflict of interests: The authors declare no conflicts of interest.

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