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Co-expression of the calcitonin receptor gene in the hypothalamic kisspeptin neurons in female rats

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Abstract

Purpose: Hypothalamic kisspeptin neurons are considered to play a critical role in regulating mammalian reproduction and integrating humoral and neuronal inputs that control gonadotropin-releasing hormone (GnRH)/gonadotropin release. The present study aimed to investigate the upstream regulator candidates for kisspeptin neurons.

Methods: Visualized kisspeptin neurons that were taken from the arcuate nucleus (ARC) of *Kiss1*-tdTomato rats were subjected to next-generation sequencing (NGS) analysis. In situ hybridization (ISH) for the calcitonin receptor gene (*Calcr*) was performed throughout the whole forebrain of ovariectomized wild-type female rats that had been implanted with a negative feedback level of estrogen, because the *Calcr* expression was evident in the ARC kisspeptin neurons from the NGS analysis. Then, a double ISH was performed for the *Calcr* and kisspeptin gene (*Kiss1*) in the brain regions, containing either the anteroventral periventricular nucleus (AVPV) or ARC of the female rats.

Results: The NGS analysis revealed that the *Calcr* was highly expressed in the ARC kisspeptin neurons. It was found that the *Calcr* was co-expressed in 12% and 22% of the *Kiss1*-expressing cells in the ARC and AVPV, respectively.

Conclusion: The present study suggests that calcitonin receptor signaling could be involved in the regulation of reproductive function through the direct control of the ARC and/or AVPV kisspeptin neurons, and then GnRH/gonadotropin release.

KEYWORDS
anteroventral periventricular nucleus, arcuate nucleus, calcitonin receptors, in situ hybridization, kisspeptin-1

1 | INTRODUCTION

Increasing evidence suggests that kisspeptin–GPR54 (kisspeptin receptor) signaling plays a critical role in the regulation of reproductive functions via the direct stimulation of gonadotropin-releasing hormone (GnRH) and then gonadotropin release in mammals, including rodents, ruminants, primates, pigs, and musk shrews.[1-6] Mutations of *Kiss1*, a gene encoding kisspeptin, or *Gpr54* cause hypogonadotropic hypogonadism

in female rodents and humans,[1,2,7-9] suggesting that kisspeptin–GPR54 signaling is indispensable for puberty and normal reproductive function at adulthood in mammals. The expression of *Kiss1* and kisspeptin has been found in two hypothalamic regions: the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) in rodents.[10-12] Both the ARC and AVPV kisspeptin neurons express estrogen receptor alpha in female rodents[10,12,13] and estrogen negatively and positively regulates *Kiss1* expression in the ARC and AVPV kisspeptin neurons, respectively.[10,12,13]

Thus, the ARC and AVPV kisspeptin neurons are considered to mediate estrogen negative and positive feedback effects on GnRH/gonadotropin release and therefore they are suggested to govern GnRH/gonadotropin pulses and surges, respectively[10,11,13] Hypothalamic kisspeptin neurons are suggested to be hub neurons that receive inputs from other neurons to mediate the influence of the exogenous or endogenous environment on reproduction through controlling GnRH release. Indeed, leptin receptors are expressed in the ARC kisspeptin neurons and these receptors are suggested to be involved in the facilitation of the ARC kisspeptin neurons under a high available nutritional status[14] because the secretion of leptin, an anorexigenic hormone from the adipose tissue, is stimulated by food intake to exert several physiological roles, such as the inhibition of feeding behavior and the facilitation of gonadotropin release.[14,15] Furthermore, the authors' previous study showed that glutamatergic agonists, which are potent stimulators for gonadotropin release, failed to stimulate luteinizing hormone (LH) release in *Kiss1* KO rats,[1] suggesting that kisspeptin neurons mediate the glutamatergic stimulation of gonadotropin release.

The present study aimed to investigate the upstream regulator candidates for the kisspeptin neurons located in the ARC. To this end, the receptors that are exclusively expressed in kisspeptin neurons were analyzed by using next-generation sequencing (NGS) on isolated visualized kisspeptin neurons that had been taken from the ARC of *Kiss1*-tdTomato heterozygous rats. In order to investigate the ligand-receptor signaling candidates that affect kisspeptin neurons, and consequently tonic GnRH/gonadotropin release, the focus was on the G-protein-coupled receptors (GPCRs) that were highly expressed in the ARC kisspeptin neurons. The NGS analysis revealed that considerable levels of calcitonin receptor (CALCR) messenger (m)RNA were expressed in the ARC kisspeptin neurons in the *Kiss1*-tdTomato female rats. Thus, the localization of *Calcr* in rat forebrains was investigated and it was examined if *Calcr* is co-expressed in the ARC, as well as in the AVPV kisspeptin neurons, in female rats by using double in situ hybridization (ISH).

2 | MATERIALS AND METHODS

2.1 | Animals

Adult female Wistar–Imamichi rats at 8-9 weeks of age (206-225 g body weight [BW]) and genetically modified rats carrying the tandem dimer Tomato (tdTomato; Clontech, Mountain View, CA, USA) in the *Kiss1* locus (*Kiss1*–tdTomato) at 12 weeks of age (244-288 g BW)[1] were maintained under a controlled environment (14 hours light and 10 hours darkness; lights on at 05:00 hours; 23 ± 3°C) with free access to food (CE2; Clea, Tokyo, Japan) and water. The vaginal smears were checked daily to determine their estrous cyclicity and females with at least two consecutive estrous cycles were used. The *Kiss1*-tdTomato heterozygous rats that had been ovariectomized (OVX) 2 weeks prior were used for the NGS analysis of the ARC kisspeptin neurons. The OVX *Kiss1*-tdTomato rats without estradiol-17β (E2) treatment were chosen because E2 treatment suppresses *Kiss1* and kisspeptin, as well as tdTomato expression in the ARC. The Wistar–Imamichi rats were OVX and then immediately received subcutaneous Silastic implants (internal

diameter: 1.57 mm; outer diameter: 3.18 mm; 25 mm in length; Dow Corning, Midland, MI, USA) that were filled with E2 (20 μg/mL in peanut oil) for 1 week to serve as the OVX + low E2 rats. The low E2 treatment was previously confirmed to produce a plasma E2 level of 35.8 pg/mL and to produce a negative feedback effect on LH pulses, but not to induce LH surges.[16] The OVX + low E2 rats were chosen so as to analyze the co-expression of the *Calcr* and *Kiss1* in both the AVPV and ARC because the OVX + low E2 rat model was suitable to detect *Kiss1* in these nuclei. All the operations were performed under ketamine (27 mg/kg) and xylazine (5.3 mg/kg) anesthesia and aseptic conditions. All the rats were injected with antibiotics (Mycillin Sol; Meiji Seika, Tokyo, Japan) after surgery. One week after the OVX and E2 treatment, the animals were deeply anesthetized with sodium pentobarbital (40 mg/kg, Kyoritsu Seiyaku Co., Tokyo, Japan) and perfused with 0.05 mol L^{-1} phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.05 mol L^{-1} PB. The brain was immediately removed from the skull, postfixed with the same solution for 6-7 hours at 4°C, and then kept in 30% sucrose in 0.05 mol L^{-1} PB for 3-4 days at 4°C under ribonuclease (RNase)-free conditions. Serial coronal sections (50 μm in thickness) of the forebrain that contained the hypothalamus and the mid-brain (from 0.0 mm to 4.2 mm posterior to the bregma) were obtained by using a cryostat on the day before ISH and then were stored at 4°C in PBS.

2.2 | Expression analysis of the G-protein-coupled receptors in isolated tdTomato-positive cells taken from *Kiss1*-tdTomato rats

The ARC tissues were dissected out from the OVX *Kiss1*-tdTomato heterozygous rats. Acutely dissociated cells were prepared, as described previously,[17] with slight modifications. Briefly, the tissues were treated with 10 U/mL papain (Worthington, Lakewood, NJ, USA) for 50 minutes at 30°C in Dulbecco's modified Eagle medium (Life Technologies, Carlsbad, CA, USA), gently dispersed by repetitive pipetting, then filtered through a 70-μm pore size nylon mesh (BD Biosciences, San Jose, CA, USA). Cell suspensions were applied to a discontinuous Percoll (Sigma-Aldrich, St. Louis, MO, USA) density gradient that was composed of 0.28 and 0.75 g/mL layers, and then centrifuged. The cells were obtained from the middle layer. The cell suspension was placed onto plastic dishes. The tdTomato-positive cells were collected under a fluorescent microscope by pipettes (inner diameter: 20-30 μm) that were filled with 150 mmol L^{-1} KCl, 3 mmol L^{-1} MgCl$_2$, 5 mmol L^{-1} EGTA, and 10 mmol L^{-1} HEPES solution. Ten positive cells were pooled into a polymerase chain reaction (PCR) tube containing a RNase inhibitor (RNasin Plus; Promega, Madison, WI, USA).

The complementary (c)DNAs were synthesized with a SMARTer Pico PCR cDNA Synthesis kit (Clontech) and amplified by universal primers in accordance with the manufacturer's instructions. The first-round PCR was performed at 94°C for one minute, then 21 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for six minutes, and finally 72°C for 10 minutes. A 100-μL aliquot of PCR mixture contained 61 μL of the template cDNA, 2 μL of 12 μmol L^{-1} cDNA synthesis primer (Takara Bio, Kusatsu, Japan), 10 μL of 25 mmol L^{-1} MgCl$_2$, 16 μL of 2.5 mmol L^{-1} dNTP mix, and 1 μL of Takara LA-taq DNA polymerase (Takara Bio). The

products of the first-round PCR were fragmented and used to generate a library for the RNA-seq. Barcoded SOLiD 3' primers from the SOLiD RNA Barcoding Kit (Life Technologies) were used to distinguish between the samples. The library was sequenced by multiplex paired-end sequencing on a flow cell using the 5500xl SOLiD system (Life Technologies). Mapping of the paired-end reads to the rat reference genome (RGSC 3.4 assembly) was performed. The mRNA expression levels were normalized by calculating the reads per kilo base per million mapped reads (RPKM) for each mRNA. The mRNAs coding for GPCR were extracted from the RNA-seq data by using the rat GPCR dataset that had been obtained from the SEVENS GPCR database (http://sevens.cbrc.jp).

2.3 | In situ hybridization for *Calcr*

In order to detect *Calcr* mRNA, a *Calcr*-specific digoxigenin (DIG)-labeled probe (position 293-1303; GenBank accession no. NM_053816) was synthesized by using a DIG-labeling kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). The sections were washed with PBS and treated with 1 μg/mL protease K for 15 minutes at 37°C. Then, they were incubated with 0.25% acetic anhydride in 0.1 mol L^{-1} triethanolamine for 10 minutes. Finally, the sections were hybridized overnight at 60°C with 1 μg/μL 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 that had been treated with a sense probe. After hybridization, the sections were washed with 2 × saline-sodium citrate (SSC) containing 50% formamide for 15 minutes at 60°C twice. The sections then were treated with 20 μg/mL RNase A for 30 minutes at 37°C and then immersed sequentially in 2 × SSC, 0.5 × SSC, and DIG-1 buffer (100 mmol L^{-1} Tris-HCl [pH = 7.5], 150 mmol L^{-1} NaCl, and 0.01% Tween20) for 15 minutes, twice each. Following this, the sections were immersed in 1.5% blocking reagent (Roche Diagnostics Corporation) in DIG-1 buffer for one hour at room temperature and incubated with an alkaline phosphatase-conjugated anti-DIG antibody (1:1000; Roche Diagnostics Corporation) overnight at 4°C. Then, the sections were washed with DIG-1 buffer and treated with DIG-3 buffer (100 mmol L^{-1} Tris-HCl [pH = 9.5], 100 mmol L^{-1} NaCl, and 50 mmol L^{-1} MgCl$_2$). Following this, the sections were treated with a chromagen solution (337 μg/mL 4-nitroblue tetrazolium chloride and 175 μg/mL 5-bromo-4-cloro-3-indolyl-phosphate in DIG-3 buffer) at 37°C until a visible signal was detected. The reaction was stopped by adding a reaction stop solution (10 mmol L^{-1} Tris-HCl [pH = 7.6] and 1 mmol L^{-1} EDTA [pH = 8.0]), then washed two times with 0.05 mol L^{-1} PBS. After processing, the sections were mounted and examined by light microscopy (BX53; Olympus, Tokyo, Japan).

2.4 | Double in situ hybridization for *Calcr* and *Kiss1*

In order to detect the *Calcr* and *Kiss1* mRNA, the *Calcr*-specific DIG-labeled cRNA probe and the *Kiss1*-specific fluorescein isothiocyanate (FITC)-labeled cRNA probe (position 33-348; GenBank accession no. AY196983) that had been synthesized from the rat hypothalamic cDNA by using a FITC-labeling kit (Roche Diagnostics)

were used. Briefly, the sections were washed with PBS, treated with 1 μg/mL protease K for 15 minutes at 37°C, and then incubated with 0.25% acetic anhydride in 0.1 mol L^{-1} triethanolamine for 10 minutes. Finally, the sections were hybridized overnight at 60°C with 1 μg/μL DIG-labeled *Calcr* anti-sense cRNA probes and 1 μg/μL FITC-labeled *Kiss1* anti-sense cRNA probes. After hybridization, the sections were washed twice with 2 × SSC containing 50% formamide for 15 minutes at 60°C. The sections then were treated with 20 μg/mL RNase A for 30 minutes at 37°C and immersed sequentially with 2 × SSC, 0.5 × SSC, and 1% H$_2$O$_2$ in 0.05 mol/L PBS for 30 minutes at 30°C. Next, the sections were washed once with 0.05 mol L^{-1} PBS and DIG-1 buffer (100 mmol L^{-1} Tris-HCl [pH = 7.5], 150 mmol L^{-1} NaCl, and 0.01% Tween-20) for five minutes. Subsequently, the sections were immersed with 1.5% blocking reagent (Roche Diagnostics) in the DIG-1 buffer for one hour at room temperature and incubated with a peroxidase (POD)-conjugated anti-FITC antibody in 1.5% blocking reagent (1:1000) for two hours at room temperature. Then, the sections were washed with DIG-1 buffer for five minutes two times and treated with tyramide signal amplification (TSA) plus FITC (1:100; PerkinElmer, Inc., Shelton, CT, USA) for 30 minutes at room temperature. The sections were observed under a fluorescence microscope in a dark room for the *Kiss1* mRNA visible signal to be detected. The reaction was stopped by washing two times with DIG-1 buffer for five minutes at room temperature, then treated with 0.1 N HCl for 15 minutes at room temperature. The sections were washed two times with DIG-1 buffer for five minutes at room temperature and incubated with a POD-conjugated anti-DIG antibody in 1.5% blocking reagent (1:1000) overnight at 4°C, then washed two times with DIG-1 buffer at room temperature and treated with TSA plus Biotin (1:100; PerkinElmer) for 30 minutes at room temperature, followed by being washed two times with DIG-1 buffer at room temperature. Subsequently, the sections were treated with Dylight 594-conjugated streptavidin (Thermo Fisher Scientific, Waltham, MA, USA) in DIG-1 buffer (1:500) for 20 minutes at room temperature. All the sections were mounted to gelatin-coated slides and the photomicrographs were taken under a fluorescence microscope by using ApoTome (Carl Zeiss, Oberkochen, Germany), where the number of *Kiss1*-expressing cells and *Calcr*-expressing cells were counted. The visualized cells throughout the AVPV (five-to-seven sections divided into two parts, from the 0.0-0.6 mm posterior to the bregma according to a rat brain atlas[18]) and ARC (10-12 sections divided into three parts, from the 1.8-4.2 mm posterior) were counted bilaterally twice by one of the authors under a microscope and the average was calculated. No positive signal for *Calcr* or *Kiss1* mRNA was detected in the brain sections that had been hybridized with the corresponding sense probes.

3 | RESULTS

3.1 | Discovery of *Calcr* expression in the arcuate kisspeptin neurons by a next-generation sequencing analysis

The RNA-seq of kisspeptin neurons that had been derived from the ARC of the OVX *Kiss1*-tdTomato heterozygous female rats (total

reads: 66,840,006; mean read length: 53 bp; aligned base rate: 88%) showed that about 8200 genes possibly were expressed in the ARC kisspeptin neurons, in terms of the RPKM values. The list included well-known genes, such as *Kiss1* (2252 RPKM), *Tac3* (encoding neurokinin B, 4384 RPKM), and *Pdyn* (encoding dynorphin,

443 RPKM). In addition, several mRNAs that encode GPCR, including *Tacr3* (encoding neurokinin 3 receptor, 199 RPKM) and *Calcr* (83 RPKM), were detected. The RPKM value of *Calcr* ranked second among the GPCR mRNAs that were expressed in the ARC *Kiss1*-tdTomato cells. No *Gpr54*, *Oprk1* (encoding dynorphin receptor),
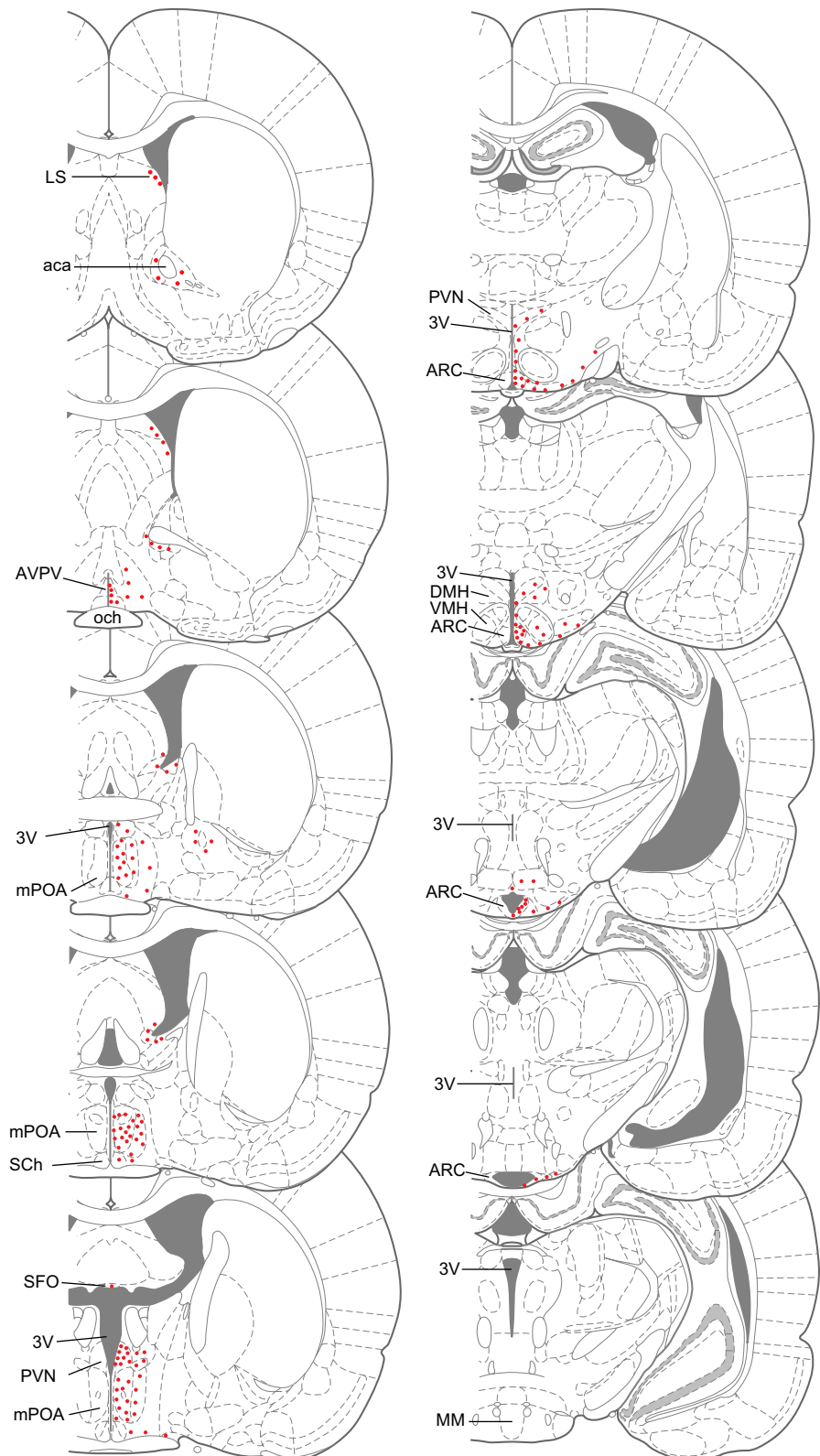

FIGURE 1 Schematic illustration showing the distribution of *Calcr* messenger RNA expression in the forebrain of ovariectomized + low estradiol rats, according to the rat brain map.[18] The *Calcr*-expressing cell bodies are indicated by red dots (one dot corresponding to 25 cells). 3V, third ventricle; aca, anterior commissure; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; DMH, dorsomedial hypothalamus; LS, lateral septum; MM, mammillary nucleus; mPOA, medial preoptic area; och, optic chiasm; PVN, paraventricular nucleus; SCh, suprachiasmatic nucleus; SFO, subfornical organ; VMH, ventromedial hypothalamic nucleus
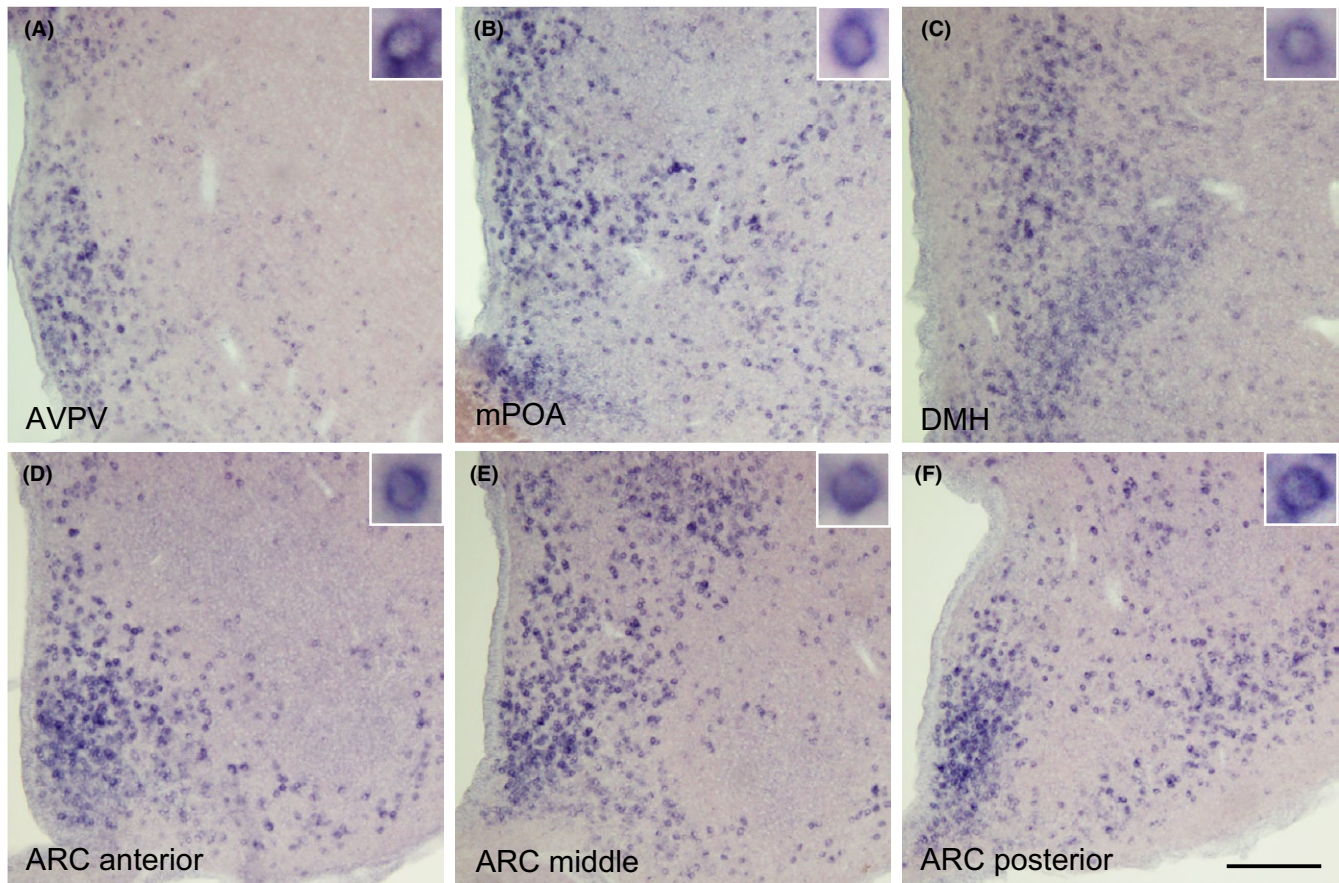
FIGURE 2 *Calcr*-expressing cells that were detected by in situ hybridization in the hypothalamus of the representative ovariectomized + low estradiol rats. The *Calcr*-expressing cells were significantly found in the (A) anteroventral periventricular nucleus (AVPV), (B) medial preoptic area (mPOA), (C) dorsomedial hypothalamus (DMH), and (D) anterior, (E) middle, and (F) posterior parts of the arcuate nucleus (ARC). The insets indicate *Calcr*-expressing cells at a higher magnification. Scale bar: 200 μm

and *Gnrhr* (encoding GnRH receptor) expression was detected in the ARC kisspeptin neurons in the rats.

3.2 | Distribution of the *Calcr*-expressing cells in the forebrain of the female rat

Figure 1 indicates a schematic illustration that shows the sites of *Calcr* mRNA expression throughout the forebrain of the OVX + low E2 rats. *Calcr*-expressing cells were found in the AVPV, ARC, medial preoptic area (mPOA), and dorsomedial hypothalamus (DMH). More specifically, a large number of *Calcr*-expressing cells were found in the whole ARC and *Calcr*-expressing cells were located abundantly in the central-to-caudal parts of the ARC. In addition, a number of *Calcr*-expressing cells were located in the region close to the cerebral ventricles, such as the lateral and third cerebroventricles.

Figure 2 shows the *Calcr* mRNA expression that was detected by ISH in the forebrain of the representative OVX + low E2 rats. A number of *Calcr*-expressing cells was found in the AVPV (Figure 2A), mPOA (Figure 2B), DMH (Figure 2C) and anterior (Figure 2D), middle (Figure 2E), and posterior of the ARC (Figure 2F).

3.3 | Colocalization of *Calcr* and *Kiss1* expression in the arcuate nucleus and anteroventral periventricular nucleus in the female rat

Figure 3 shows the colocalization of *Calcr and Kiss1* mRNA expression in the ARC and AVPV of the representative OVX + low E2 rats. *Calcr* expression was found in some *Kiss1*-expressing cells in the ARC (Figure 3A-C). *Calcr* expression also was detected in a part of the *Kiss1*-expressing cells in the AVPV (Figure 3D-F). Figure 4 shows the quantitative analysis of the *Kiss1*- and *Calcr*-expressing cells in the ARC and AVPV in the OVX + low E2 rats (n = 4; note that the y-axis is expressed as a logarithmic scale). It was found that some *Kiss1*-expressing cells that were located in the rostral-to-caudal parts of the ARC and AVPV co-expressed *Calcr*; similarly, a part of the *Calcr*-expressing cells co-expressed *Kiss1*. In total, 12% of the ARC *Kiss1*-expressing cells showed *Calcr* co-expression, while 35% of the *Calcr*-expressing cells in the ARC showed co-expression of *Kiss1* (Figure 4A,B); 22% of the AVPV *Kiss1*-expressing cells showed *Calcr* mRNA expression, while 12% of the AVPV *Calcr*-expressing cells showed co-expression of *Kiss1*.
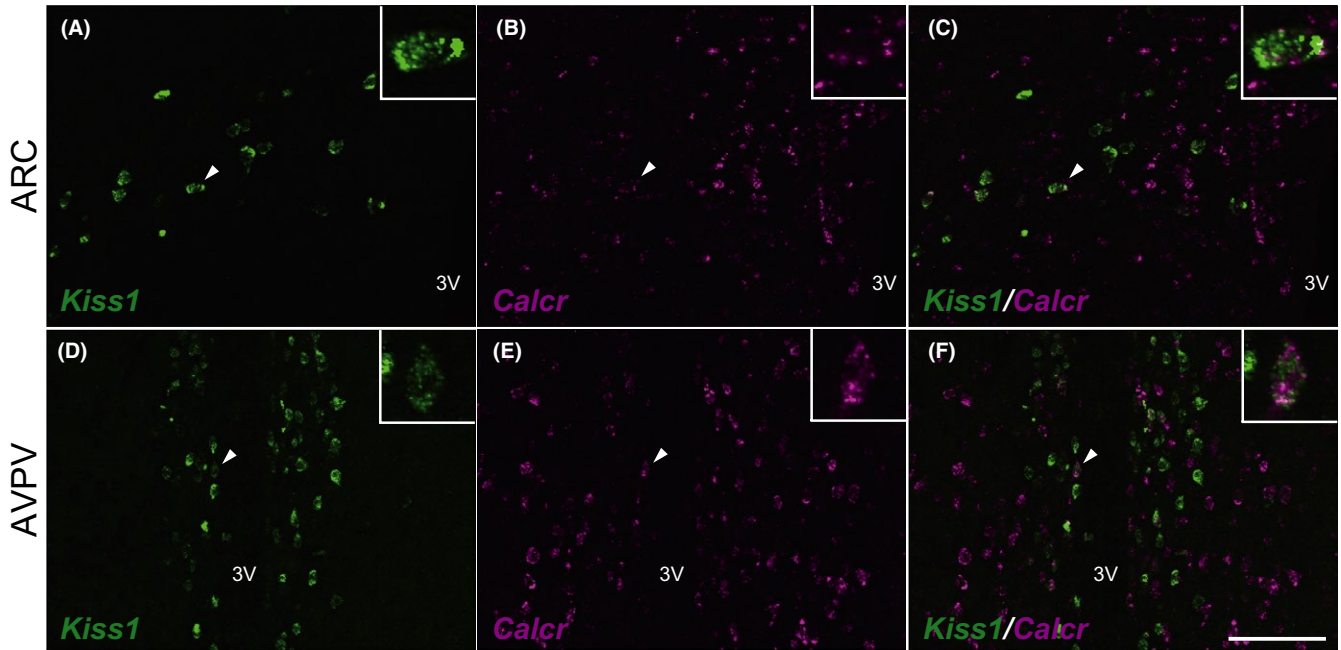
FIGURE 3 *Kiss1* (green)- and *Calcr* (magenta)-expressing cells in the arcuate nucleus (ARC) (A-C) or anteroventral periventricular nucleus (AVPV) (D-F) in the representative ovariectomized + low estradiol rats. The insets indicate the *Kiss1*- and *Calcr*-co-expressing cells (arrowheads) at a higher magnification. Scale bar: 100 μm. 3V, third ventricle
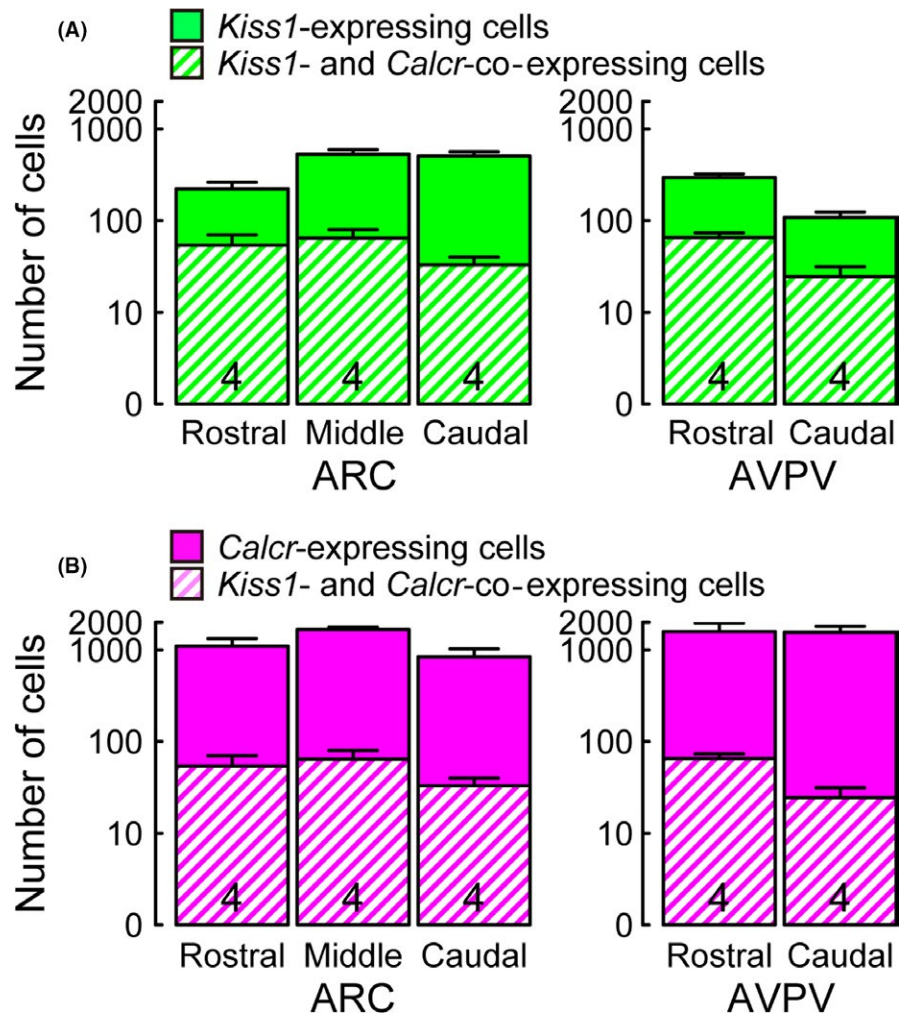
FIGURE 4 Numbers of *Kiss1*- and *Calcr*-expressing cells in the ARC or AVPV. The number of *Kiss1*-expressing cells with or without *Calcr* expression in the ARC or AVPV in OVX+low E2 rats (A). The number of *Calcr*-expressing cells with or without *Kiss1* expression in the ARC or AVPV in OVX + low E2 rats (B). Values are mean ± SEM and are represented in a logarithmic scale on the Y-axis. Numbers in each column indicate the number of animals used. The ARC was divided into three parts (from 1.8 to 2.6 mm, from 2.6 mm to 3.4 mm and from 3.4 to 4.2 mm posterior to the bregma) while the AVPV was divided into two parts (from 0.0 to 0.3 mm and from 0.3 to 0.6 mm posterior to the bregma)

4 | DISCUSSION

In the present study, the NGS analysis revealed that considerable levels of *Calcr* are expressed in the ARC kisspeptin neurons. The further histological analysis with double ISH for *Calcr* and *Kiss1* revealed that *Calcr* was co-expressed in a part of the ARC *Kiss1*-expressing cells, as well as the AVPV *Kiss1*-expressing cells. Kisspeptin neurons have been well accepted to be a master regulator for reproduction via stimulation of GnRH and the consequent gonadotropin release in mammals.[1-6] The ARC kisspeptin neurons are considered to be involved in GnRH/gonadotropin pulse generation, which is involved in folliculogenesis, spermatogenesis, and steroidogenesis, while AVPV kisspeptin neurons are considered to be responsible for GnRH/LH surge generation, which in turn regulates ovulation. Thus, CALCR signaling could have a role in affecting kisspeptin neurons in the ARC and/or AVPV to regulate GnRH/gonadotropin pulses and/or surges, respectively. In addition to the AVPV and ARC, *Calcr* was observed in several regions of the hypothalamus, such as the mPOA, DMH, and regions close to the cerebral ventricles, such as the lateral and third cerobroventricles. The results are largely consistent with previous reports in rats using ISH and immunohistochemistry to detect *Calcr* and CALCR.[19,20]

The CALCR is a member of a subfamily of the seven-transmembrane domain GPCR superfamily. Calcitonin, an endogenous ligand for CALCR[21] that is secreted from parafollicular cells of the thyroid gland, is known to be involved in regulating peripheral calcium metabolism during pregnancy and lactation in mammals[22-24] to protect the maternal skeleton by direct action on osteocytes to inhibit osteolysis.[24-26] Interestingly, previous studies demonstrated that a peripheral administration of calcitonin decreased the plasma LH and testosterone levels in rats.[27,28] Furthermore, a central injection of a calcitonin family peptide hormone, such as calcitonin gene-related peptide, suppressed LH pulses in female rats.[29] It is reported that LH release is suppressed during lactation in rats and cows.[30-33] These results suggest that calcitonin–CALCR signaling in hypothalamic kisspeptin neurons could be involved in the suppression of LH release in lactating animals, in which calcium availability for milk synthesis is essential,[24] and plasma calcitonin levels increase in humans and rats.[22,26,34] Thus, it is speculated that calcium availability could affect the reproductive function in mammals via the effect of calcitonin–CALCR signaling on the ARC and/or AVPV kisspeptin neurons.

Amylin has been demonstrated as another endogenous ligand for CALCR and it was originally found as a product in the pancreatic B cells. It is coreleased with insulin under hyperglycemic conditions or after feeding.[35] Amylin expression is also evident in the brain in rats.[36] Amylin is known to be capable of binding to CALCR, when it forms a complex with receptor activity-modifying proteins.[37,38] Thus, amylin that was derived in the peripheral organs and/or brain could be involved in regulating GnRH/LH release via CALCR that is expressed in the hypothalamic kisspeptin neurons. It is demonstrated that a central injection of amylin inhibits sexual behavior in male rats.[39] Interestingly, amylin mRNA levels increased in the mPOA in rats at lactation,[40] during which LH release was profoundly suppressed. Taken together with the present study, these results suggest that calcitonin or amylin could exert an inhibitory role in GnRH/gonadotropin release via the activation of CALCR in the ARC and AVPV kisspeptin neurons.

The present study showed that a large population of the ARC *Kiss1*-expressing cells did not express *Calcr*. The ARC *Kiss1*-expressing cells without *Calcr* could be affected indirectly via the neighboring *Kiss1*-expressing cells because the authors' previous study showed that kisspeptin neurons in the ARC interact with each other via gap junctional connections in mice.[41] A number of *Calcr*-expressing cells without *Kiss1* expression in the ARC also was found. Those cells might be dopaminergic cells because the central and peripheral administration of calcitonin decreased the plasma prolactin levels in the lactating rats.[42] Thus, it is speculated that calcitonin and/or amylin could affect the tubuloinfundibular dopaminergic (TIDA) neurons, which in turn, might contribute to controlling prolactin release from the anterior pituitary during lactation. Interestingly, GPR54 expression was evident in the TIDA neurons in rats,[43] suggesting that ARC kisspeptin and TIDA neurons could interact with each other through kisspeptin and CALCR signaling. Furthermore, the *Calcr*-expressing cells in the brain regions close to the cerebral ventricles might have a role as an interface of peripheral and central interaction. Indeed, the circulating calcitonin is capable of binding to the CALCR around the ventricles.[44] Further studies are required to clarify the role of the CALCR around the ventricles.

In conclusion, the present study suggests that the ARC and AVPV kisspeptin neurons could be direct action sites of calcitonin and/or amylin and that the CALCR signaling might have a role in regulating GnRH/gonadotropin release because *Calcr* expression was evident in some of the kisspeptin neurons in these nuclei. Further studies are required to clarify if the CALCR signaling exerts inhibitory regulation of GnRH/gonadotropin release.

DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. *Human rights statement and informed consent*: This article does not contain any study with human participants that was performed by any of the authors. *Animal studies:* All the institutional and national guidelines for the care and use of laboratory animals were followed. All the experiments were approved and conducted in accordance with the guidelines of the Committee of Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan.

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