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ORIGINAL PAPER

Region-specific changes in brain kisspeptin receptor expression during estrogen depletion and the estrous cycle

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Abstract

Kisspeptin acts as a potent neuropeptide regulator of reproduction through modulation of the hypothalamic–pituitary–gonadal axis. Previous studies revealed sex differences in brain expression patterns as well as regulation of expression by estrogen. Alternatively, sex differences and estrogen regulation of the kisspeptin receptor (encoded by *Kiss1r*) have not been examined at cellular resolution. In the current study, we examined whether *Kiss1r* mRNA expression also exhibits estrogen sensitivity and sex-dependent differences using in situ hybridization. We compared *Kiss1r* mRNA expression between ovariectomized (OVX) rats and estradiol (E2)-replenished OVX rats to examine estrogen sensitivity, and compared expression between gonadally intact male rats and female rats in diestrus or proestrus to examine sex differences. In OVX rats, E2 replenishment significantly reduced *Kiss1r* expression specifically in the hypothalamic arcuate nucleus (ARC). A difference in *Kiss1r* expression was also observed between diestrus and proestrus rats in the hypothalamic paraventricular nucleus (PVN), but not in the ARC. Thus, estrogen appears to have region- and context-specific effects on *Kiss1r* expression. However, immunostaining revealed minimal colocalization of estrogen receptor alpha (ERα) in *Kiss1r*-expressing neuronal populations of ARC and PVN, indicating indirect or ERα-independent regulation of *Kiss1r* expression. Surprisingly, unlike the kisspeptin ligand, no sexual dimorphisms were observed in either the brain distribution of *Kiss1r* expression or in the number of *Kiss1r*-expressing neurons within enriched brain nuclei. The current study reveals marked differences in regulation between kisspeptin and kisspeptin receptor, and provides an essential foundation for further study of kisspeptin signaling and function in reproduction.

Keywords Kisspeptin receptor · GPR54 · Sex difference · Estrogen sensitivity

Introduction

Kisspeptin–kisspeptin receptor signaling is of key importance for fertility in mammals. Kisspeptin is a family of four peptides (kisspeptin-54, -14, -13, and -10) generated by proteolytic cleavage from a common precursor protein encoded by the *KISS1*/*Kiss1* gene. All kisspeptins bind to and efficiently activate the G protein-coupled receptor KISS1R (also known as GPR54) (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). Kisspeptin stimulates gonadotropin-releasing hormone (GnRH) secretion via KISS1R on GnRH neurons in the hypothalamus. Through GnRH secretion, kisspeptin controls activity of the hypothalamic–pituitary–gonadal (HPG) axis, and consequently regulates multiple aspects of reproductive system function, including onset of puberty, the estrous cycle, and spermatogenesis (Clarke et al. 2015; Popa et al. 2008; Liu and Herbison 2016). Since kisspeptin is an important upstream regulator of the HPG axis, modulation of the kisspeptin system has been studied in various reproductive conditions. Sex differences in brain kisspeptin expression and distribution have been reported, and dynamic changes have been observed in response to changes in brain estrogen (Takumi et al. 2011; Adachi et al. 2007; Kauffman et al. 2007; Smith et al. 2005a, b, 2006; Navarro et al. 2004).

Kisspeptin-expressing neurons are mainly located in two discrete hypothalamic regions, the rostral periventricular area

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of the third ventricle (RP3V) and the arcuate nucleus (ARC) (Smith et al. 2006; Irwig et al. 2004; Gottsch et al. 2004). Kisspeptin expression in the RP3V and ARC are reciprocally regulated by estrogen; estrogen upregulates kisspeptin in the RP3V and downregulates kisspeptin in the ARC (García-Galiano et al. 2012).

In contrast to this rich knowledge on kisspeptin ligand expression, little is known of the physiological regulation and sex differences in receptor expression. In particular, no detailed histological data has been reported, probably due to the low levels of *Kiss1r* expression and lack of reliable KISS1R antibodies. A few reports using RT-PCR assays have suggested sexual dimorphism in *Kiss1r* expression as well as estrogen sensitivity; however, there are some inconsistencies. Navarro et al. reported hormonal regulation of *Kiss1r* mRNA expression patterns in the rat hypothalamus (Navarro et al. 2004), whereas Adachi et al. reported relatively stable *Kiss1r* expressions across the estrous cycle and no clear sex differences in the anteroventral periventricular area of the hypothalamus (Adachi et al. 2007). These inconsistencies may partially reflect the lack of spatial information provided by PCR analysis; therefore, detailed histological mapping of expression is needed.

Recently, we succeeded in visualizing the expression of *Kiss1r* using in situ hybridization (ISH) and mapped the distribution of *Kiss1r* mRNA in the female rat brain (Higo et al. 2016). Using this method, the present study aimed to examine estrogen sensitivity and sexual dimorphism in *Kiss1r* expression.

To assess the effect of estrogen on *Kiss1r* expression in rat brain, we compared anatomic expression patterns between ovariectomized (OVX) and estradiol (E2)-replenished OVX rats (OVX + high-E2) using ISH. Subsequently, we counted the numbers of ISH-positive neurons in the three brain areas showing highest *Kiss1r* expression, a rostral area including the medial septum and diagonal band of Broca (hereinafter, referred to as "DB"), the paraventricular nucleus of the hypothalamus (PVN), and the ARC (Higo et al. 2016). To reveal sexual differences and changes in *Kiss1r* expression during the estrous cycle, we compared expression patterns among gonadally intact males and females at different stages of the estrous cycle. We also examined colocalization of estrogen receptor alpha (ERα) in the *Kiss1r*-expressing neuronal populations of each area: GnRH neurons in the DB, oxytocin neurons in the PVN (Higo et al. 2016), and pro-opiomelanocortin (POMC) neurons in the ARC (Higo et al. 2017).

Materials and methods

Animals

Adult female and male Wistar rats (10–12 weeks old) were purchased from Tokyo laboratory animal Science (Tokyo, Japan) and were housed under controlled temperature (24 ± 2 °C) and a 14 h light/10 h dark cycle (lights on from 06:00 to 20:00) with unrestricted access to food and water. The cytology of vaginal smears was checked for all females, and those displaying at least two consecutive normal 4-day estrous cycles were used for the experiments. For gonadally intact animals, brain samples from male ($n=6$), diestrus day 1 ($n=6$), and proestrus ($n=6$) rats were obtained between 10:00 am and 13:00. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Committee on the Ethics of Animal Experiments of the Nippon Medical School approved the protocol (permit number 27–198).

Estradiol treatment

To assess the estrogen sensitivity of rat brain *Kiss1r* expression, rats were ovariectomized according to the method of Tsukamura et al. (1988), because this well-established method commonly used in the reproductive physiology would be appropriate considering the distinctive role of kisspeptin-KISS1R system in regulation of reproduction. First, female rats were randomly assigned to two groups: ovariectomized (OVX, $n=7$) and OVX with replenishment of high-dose E2 (OVX + high-E2, $n=7$). Ovariectomy and E2 treatment were performed under isoflurane inhalational anesthesia. For the OVX group, female rats were bilaterally ovariectomized via abdominal incisions at 14 days before brain tissue sampling. For the OVX + high-E2 group, female rats were ovariectomized and, then, immediately subjected to subcutaneous implantation of a silastic tube (1.0-mm inner diameter; 1.5-mm outer diameter; 20 mm in length; Dow Corning, MI, USA) filled with crystalline 17-β estradiol (E2, Sigma–Aldrich, St Louis, MO, USA) into the scapular region to produce a positive-feedback level of plasma E2. Brains were removed 2 days later for ISH and other treatments. The OVX + high-E2 group is a model for high E2 concentration, defined as a level sufficient to induce daily LH surges in OVX rats (Tsukamura et al. 1988).

Preparation of brain tissue samples

After deep anesthesia with a mixture of sodium pentobarbital (50 mg/kg, i.p.) and medetomidine hydrochloride (0.5 mg/kg, i.p.), rats were transcardially perfused with 50 mL saline

followed by 200 mL fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were post-fixed with the same fixative at 4 °C for 24 h, immersed in 20% sucrose in PB for 48 h for cryoprotection, and then frozen in pre-chilled n-hexane at − 80 °C. Serial coronal sections were cut on a cryostat (Leica CM3050, Heidelberg, Germany) at 50 µm thickness and collected in phosphate buffered saline (PBS; 0.1 M phosphate buffer, 0.9% NaCl, pH 7.4).

In situ hybridization

In situ hybridization for *Kiss1r* was performed as described previously (Higo et al. 2016). In brief, free-floating brain sections were incubated with proteinase K (1 µg/mL, Takara Bio, Otsu, Japan) in 10 mM Tris buffer (pH 7.4) and 10 mM EDTA for 25 min at 37 °C and then placed in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 15 min at room temperature (RT). After two PBS washes, the sections were hybridized with digoxigenin (DIG)-labeled cRNA probes for *Kiss1r* (positions 167–641 and 622–1185 according to NCBI reference sequence NM_023992, each at 0.5 µg/mL) in hybridization buffer [1 × hybridization solution (Sigma–Aldrich, St. Louis, MO, USA) containing 50% formamide and 10% dextran sulfate] for 16 h at 65 °C. After hybridization, the sections were rinsed in 4 × saline sodium citrate (SSC: 150 mM NaCl and 15 mM sodium citrate) containing 50% formamide followed by 2 × SSC for 20 min at 65 °C, then treated with RNaseA [20 µg/mL in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 500 nM NaCl] for 20 min at 37 °C. Then, the sections were washed under conditions of increasing stringency, followed by incubation with alkaline phosphatase (AP)-conjugated anti-DIG antibody (1:1000, Roche Diagnostics, Mannheim, Germany) for 2 h at 37 °C. Visualization of DIG-labeled probes was performed by incubation with NBT/BCIP (Roche Diagnostics) as a chromogen in Tris-based buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) under darkness for 24 h at 25 °C. Finally, after several washes in PBS, the sections were mounted on slide glass using CC/Mount mounting medium (Cosmo bio, Tokyo, Japan). For the negative control, we confirmed the absence of non-specific signals in both the sections hybridized with non-labeled antisense probes and RNase-treated sections [200 µg/mL in 10 mM Tris buffer (pH 8.0) for 30 min at 37C before the acetylation reaction] (online resource 1).

Kiss1r-positive cell counting

Images of each section were acquired using a light microscope (BX-51, Olympus, Tokyo, Japan) with a 10 × or 20 × objective lens (10×/0.40 UPlanSapo; 20×/0.75 UPlanSapo). Images were acquired using a digital camera (DP-73, Olympus, Tokyo, Japan) and image acquisition software (CellSense standard 1.6, Olympus, Tokyo, Japan). The rostro-caudal levels of each section were determined by comparing to figures in the rat brain atlas of Paxinos and Watson (Paxinos et al. 2009). The number of *Kiss1r*-positive cells was counted in the DB (approximately 1.8 to 0.48 mm anterior from bregma), PVN (approximately 1.08 to 2.04 mm posterior from bregma), and ARC (approximately 1.72 to 3.72 mm posterior from bregma) using ImageJ with the Cell Counter plugin in the gray scale mode (v1.47, NIH, Bethesda, MD, USA). The total number of *Kiss1r*-positive cells in each section series was determined by an analyzer blind to the experimental group. The mean numbers of *Kiss1r* mRNA-expressing cells with standard errors were calculated for each group.

Indirect colocalization of KISS1R and ERα by neuropeptide immunostaining

The ISH procedure causes a steep reduction in immunoreactivity, while immunostaining of KISS1R is limited by relatively low expression and antibody unreliability, thereby precluding direct visualization of KISS1R/ERα co-expression. Therefore, we examined KISS1R/ERα colocalization by co-immunostaining for ERα and the following neuropeptides known to be expressed predominantly in each area showing high *Kiss1r* expression: GnRH in the DB, oxytocin in the PVN, and POMC in the ARC. A series of serial sections from four rats from each experimental group were used for the co-expression analysis. Brain sections from the rest of rats in each experimental groups were used for the optimization of staining conditions for three combinations of antibodies used in the analysis.

For GnRH and oxytocin labeling, sections were first blocked in 2% bovine serum albumin in PBST (0.1 M phosphate buffer containing 0.9% NaCl and 0.3% Triton X-100) for 0.5 h, then, incubated with a mixture of rabbit anti-ERα polyclonal antibody (1:5000; MC-20 Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti-GnRH monoclonal antibody (1:5000; LRH13, gifted from Dr. M.K. Park, University of Tokyo, Japan) or mouse anti-oxytocin polyclonal antibody (1:5000; ab78364; Cambridge, UK) in PBST for 16 h at 4 °C. The sections were washed three times in PBST and, then, incubated with a secondary antibody mixture of Alexa Fluor 568-labeled donkey anti-rabbit IgG (1: 500, A10042; Invitrogen, Grand Island, NE, USA) and Alexa Fluor 488-labeled donkey anti-mouse IgG (1:500, A-21202, Invitrogen) for 1.5 h. Images of each section were acquired using a FV10i confocal microscope (Olympus, Tokyo, Japan) with 10 × objective lenses (UPLSAP) and FV10i software (Olympus, Tokyo, Japan).

For detection of POMC and ERα, we performed double-labeling using two sequential immunoperoxidase reactions

and the streptavidin–biotin (SAB) method (Histofine SAB-PO(R) kit, Nichirei Corporation, Tokyo, Japan) with and without nickel–cobalt enhancement instead of immunofluorescence because available antibodies for POMC and ERα are both raised in rabbits. The sections were first incubated in 10% normal goat serum at RT for 30 min and then with an anti-ERα antibody (MC-20, 1:5,000) in 10% normal goat serum at 4 °C for 16 h. The sections were washed three times in PBST and, then, were incubated with biotinylated secondary antibody at RT for 1 h, followed by three washes in PBST. Then, the sections were incubated with streptavidin-labeled peroxidase at RT for 30 min and washed three times in PBS. The ERα immunoreactivity was visualized by incubating brain sections in a chromogen solution containing diaminobenzidine (DAB; DAKO, Inc., Carpinteria, CA, USA), 0.02% nickel sulfite, and 0.001% cobalt chloride with H_2O_2 in 0.05 M Tris–HCl (pH 7.5) for 1 min, which yields an intense black stain. After three PBS washes, the sections were treated with 0.3% H_2O_2 in PBS to quench residual peroxidase activity, washed three times in PBS, and incubated with rabbit anti-POMC polyclonal antibody (dilution 1:10,000; ab94446; Abcam, Cambridge, UK) in PBST at 4 °C for 16 h. Then, the sections were then treated using the Histofine SAB-PO(R) kit and DAB without nickel and cobalt salt, yielding normal brown peroxidase staining. The sections were dehydrated and mounted with Permount (Thermo Fisher Scientific, Tokyo, Japan). A light microscope (BX-51, Olympus, Tokyo, Japan) with 20 × objective lenses (20×/0.75 UPlanSapo) was used to acquire images of each section. Images were acquired using a digital camera (DP-73, Olympus, Tokyo, Japan) and image acquisition software (CellSense standard 1.6, Olympus, Tokyo, Japan).

Peptide-immunoreactive cell counting

To estimate the cellular colocalization of ERα with GnRH, oxytocin, or POMC in each area, cell counting was performed using ImageJ with the Cell Counter plugin. For DB, three serial sections were selected from a series of brain sections of each rat at 0.2-mm intervals beginning approximately 1.32 mm anterior to bregma. For PVN, three serial sections were selected at 0.2-mm intervals beginning approximately 1.08 mm posterior to bregma. For POMC, four serial sections were selected at 0.4-mm intervals beginning approximately 1.72 mm posterior to bregma. Data from four rats were averaged and presented as the mean ± SEM.

Antibodies

Antibodies used in the present study summarized in Table 1. The primary antibodies have been well characterized by previous studies or by the manufacturer using antigen preabsorption or Western blotting. Studies providing the validation data for each antibody are follows: ERα (Bollig-Fischer et al. 2012), GnRH (Park and Wakabayashi 1986), oxytocin (Kania et al. 2017), and POMC (Konishi et al. 2015). Oxytocin validation data were also provided by the manufacturer (Merck Millipore). Immunohistochemical images obtained using these antibodies in each anatomical locus were in good agreement with previous studies and no nonspecific signals were observed in primary antibody-omitted controls.

Statistical analysis

Student's *t* test was applied for the comparison OVX and OVX + high-E2 groups, while one-way ANOVA followed by Bonferroni post hoc tests was applied for the comparison among male, diestrus, and proestrus groups. SPSS 20 for Windows software was used to conduct all the analyses. A *p* value less than 0.05 (two-tailed) was considered significant for all the tests.

Results

Kiss1r mRNA expression in OVX and OVX + high-E2 group rats

Clusters of neuronal cell bodies expressing *Kiss1r* mRNA were found throughout the brain in both OVX and OVX + high E2 groups as we reported previously (Higo et al. 2016). Strong signals were observed in several rostral brain areas, including the olfactory bulb, medial septum, DB, and throughout the preoptic area, moderate signals in the rostral periventricular area, PVN, and throughout the ARC, and relatively weak signals in the supraoptic nucleus and supramammillary nuclei. The gross anatomic distribution of

Table 1 Primary antibodies used in immunostaining

Antibody	Host	Clone	Supplier	Code	Dilution	References
ERα	Rabbit	–	Santa Cruz Biotechnology, Dallas, TX, USA	MC-20	1:5000	Bollig-Fischer et al. (2012)
GnRH	Mouse	LRH13	Gift from Dr. Park, University of Tokyo, Japan	–	1:5000	Park and Wakabayashi (1986)
Oxytocin	Mouse	–	Abcam, Cambridge, UK	ab78364	1:5000	Kania et al. (2017)
POMC	Rabbit	–	Abcam, Cambridge, UK	ab94446	1:10,000	Konishi et al. (2015)

Kiss1r mRNA expression did not appear to differ between groups; therefore, we assessed the level of expression by counting the numbers of cells in the three main expression areas: DB, PVN, and ARC. Rostro-caudal levels of these analytical areas are shown in framed panels in Fig. 1a (Nissl stain).

In the DB, the mean number of identifiable *Kiss1r*-expressing cells did not differ significantly between OVX and OVX + high-E2 groups (OVX: 24 ± 2.8, OVX + high-E2: 25 ± 3.3) (Fig. 1). Similarly, the mean number of *Kiss1r*-expressing cells in the PVN did not differ significantly between groups (OVX: 18 ± 1.6, OVX + high-E2: 15 ± 1.2). In contrast, the mean number of *Kiss1r*-expressing cells in the ARC was significantly higher in the OVX group (OVX 60 ± 5.3 vs. OVX + high-E2 24 ± 1.9; $p < 0.05$), suggesting that estrogen downregulates *Kiss1r* in a region-specific manner.

Kiss1r mRNA expression in male, diestrus, and proestrus groups

Neuronal cell bodies expressing *Kiss1r* mRNA were observed in the same anatomical loci described previously in diestrus female rats (Higo et al. 2016); therefore, we again compared mean numbers in DB, PVN, and ARC. There were no differences in the mean number of *Kiss1r*-expressing cells among the three groups in either the DB (male 38 ± 5.5, diestrus 30 ± 4.4, proestrus 33 ± 4.2) or ARC (male 68 ± 5.3, diestrus 77 ± 5.0, proestrus 65 ± 4.0) (Fig. 2). In contrast to OVX rats and OVX + high-E2 rats, no differences in mean number of *Kiss1r*-expressing ARC cells were observed among male, proestrus, and diestrus groups. On the other hand, the mean number of *Kiss1r*-expressing cells in the PVN differed significantly between diestrus and proestrus groups ($p < 0.05$), but not between male and either female group (male 22 ± 3.4, diestrus 17 ± 1.8, proestrus 30 ± 3.8). Thus, although there is a clear sex difference in kisspeptin ligand expression in the brain (Adachi et al. 2007; Kauffman et al. 2007; Takumi et al. 2011), we found no apparent difference in *Kiss1r* expression pattern or numbers of *Kiss1r*-expressing cells in the DB, PVN, and ARC between gonadally intact males and females.

Colocalization of ERα and GnRH, oxytocin, or POMC

To examine the possibility that estrogen modulates *Kiss1r* expression directly via the estrogen receptor on *Kiss1r*-expressing neurons, we examined the colocalization of *Kiss1r* with ERα in DB, PVN, and ARC. Photomicrographs taken from diestrus female rats group are shown as representative images in Fig. 3. The vast majority of *Kiss1r*-expressing neurons in rostral brain areas are

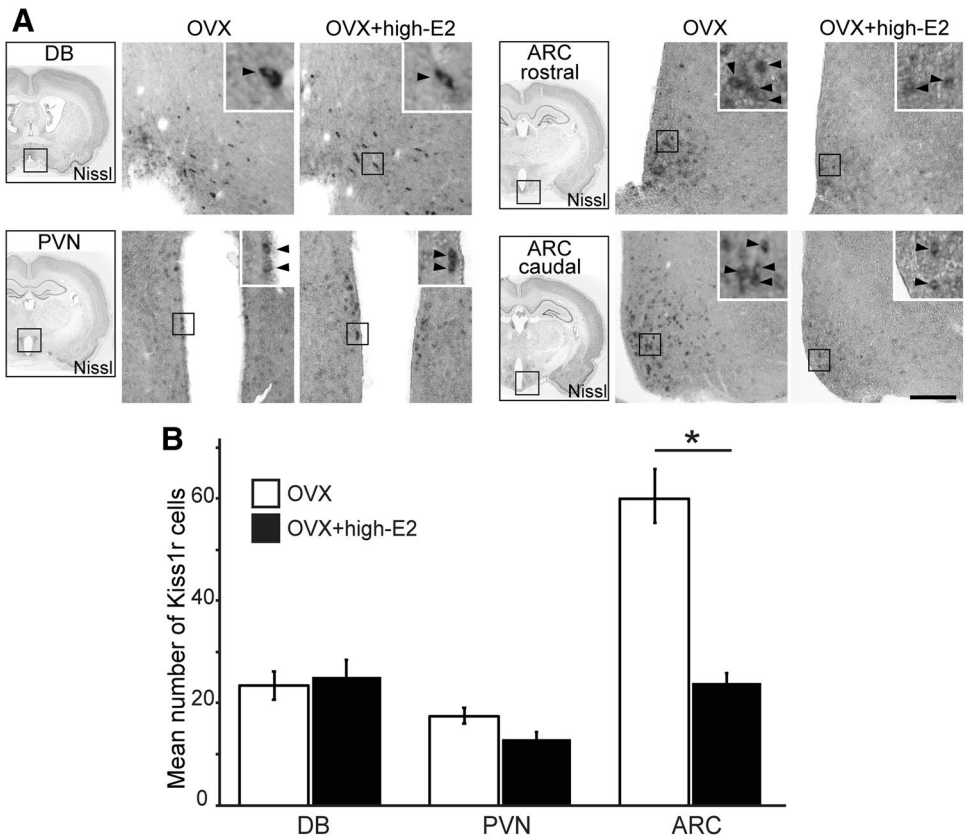

Fig. 1 *Kiss1r* mRNA expression in OVX and OVX + high-E2 group. **a** Representative photomicrographs of *Kiss1r* mRNA-expressing neurons in the diagonal band of Broca (DB), paraventricular nucleus of the hypothalamus (PVN), and arcuate nucleus of hypothalamus (ARC) of OVX and OVX + high-E2 rats. Framed low-magnification images show the rostro-caudal levels in each anatomical locus (Nissl stain). The insets shows higher magnification of each panel, and arrowheads in the insets indicate *Kiss1r*-expressing neurons. Scale bar = 200 µm. **b** Quantification of *Kiss1r* mRNA-expressing neurons. Open and solid columns represent OVX and OVX + high-E2 groups, respectively. Asterisk indicates significant difference between groups ($p < 0.05$, Student's *t* test, $n = 7$ for each group)

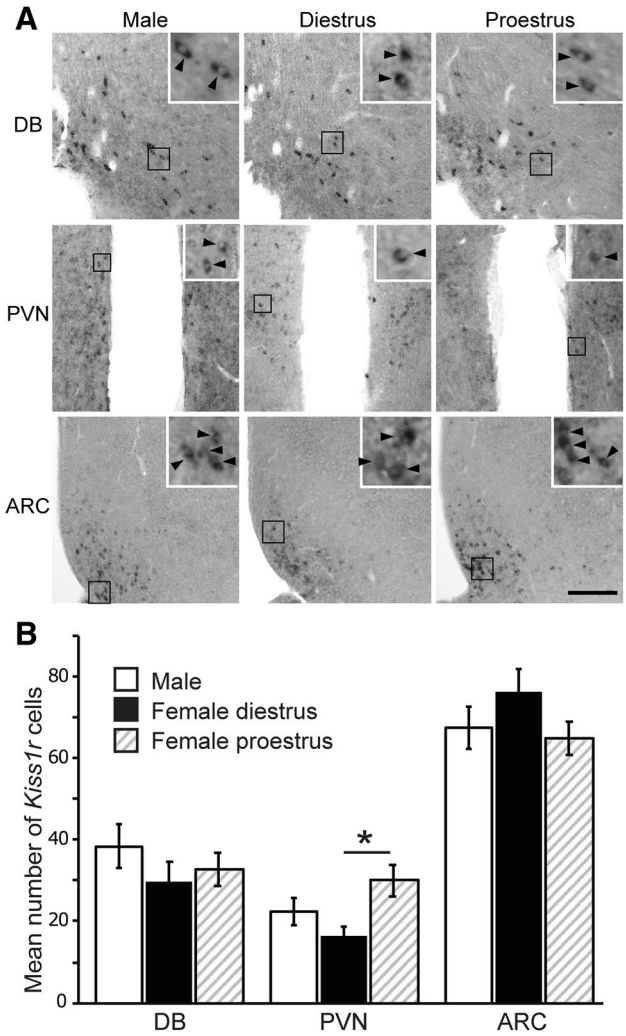

Fig. 2 *Kiss1r* mRNA expression in male, diestrus, and proestrus rats. **a** Representative photomicrographs of *Kiss1r* mRNA-expressing neurons in the DB, PVN, and ARC of male, diestrus female, and proestrus female rats. Scale bar = 200 µm. **b** Quantification of *Kiss1r* mRNA-expressing neurons. Open, solid, and hatched columns represent male, diestrus, and proestrus rats, respectively. Asterisk indicates significant difference between groups ($p < 0.05$, one-way ANOVA followed by Bonferroni post hoc test, $n = 6$ for each group)

GnRH-immunoreactive (Higo et al. 2016). In the DB, where no difference in *Kiss1r* expression was observed between OVX and OVX + high-E2 groups, there was also no measurable colocalization of GnRH and ERα in male, diestrus, and proestrus rats (Fig. 3A-a, b). In the PVN, a proportion of *Kiss1r* mRNA-expressing neurons are oxytocin-immunoreactive (Higo et al. 2016). Although *Kiss1r* expression level differed between diestrus and proestrus, immunofluorescence revealed almost no colocalization of ERα (male: 0.12% ± 0.12%, diestrus: 0.24% ± 0.24%, and proestrus 0.36% ± 0.28%) (Fig. 3B-a–c). Note that intensities of ERα signals were adjusted proportionally to the area showing the strongest signals as a reference (Fig. 3A-c for GnRH, Fig. 3B-d for oxytocin neurons). The majority (approximately 63%) of *Kiss1r* mRNA-expressing neurons in the ARC are POMC immunoreactive (Higo et al. 2017). Since both antibodies for POMC and ERα were rabbit-derived, double-label immunohistochemistry (IHC) was performed. Only a small proportion of POMC-positive neurons (showing normal brown DAB staining) co-expressed ERα as indicated by black nickel–cobalt enhanced DAB staining (male: 11% ± 1.3%, diestrus: 13% ± 2.3%, proestrus: 16% ± 2.6%, OVX: 10% ± 0.9%, OVX + high-E2: 14% ± 1.9%) (Fig. 3C-a–d). There was no significant difference between OVX and OVX + high-E2 groups (Student's *t* test) or among male, diestrus, and proestrus groups (one-way ANOVA). Notably, ERα colocalization was not higher in regions showing apparent estrogen-dependent changes in *Kiss1r* mRNA-expressing neurons.

Discussion

The purpose of this study was to examine possible estrogen sensitivity and sex differences in *Kiss1r* expression across the rat brain with fine spatial resolution. In situ hybridization revealed no sex differences in the gross regional distribution of *Kiss1r*-expressing neurons or significant differences in the numbers of *Kiss1r*-expressing neurons in regions with enriched expression (DB, PVN, and ARC). However, there were more subtle context-dependent differences. High-E2 treatment substantially reduced *Kiss1r* expression specifically in the ARC, while a change in *Kiss1r* expression across the estrous cycle was observed in the PVN. Furthermore, there was little overlap between *Kiss1r* expression and ERα expression. Thus, estrogen does not appear to directly modulate *Kiss1r* expression.

In the rodent brain, neuronal populations expressing kisspeptin are located mainly in the RP3V and ARC (Gottsch et al. 2004; Irwig et al. 2004; Smith et al. 2006). It is now widely accepted that kisspeptin expression in these two populations are reciprocally regulated by estrogen, with estrogen increasing kisspeptin expression in the RP3V and suppressing kisspeptin expression in the ARC (García-Galiano et al. 2012). However, there is limited knowledge on estrogen regulation of the kisspeptin receptor expression. Using RT-PCR, Adachi et al. (2007) reported that ARC *Kiss1r* level was slightly lower during estrus than diestrus and proestrus, and significantly lower in high E2-treated OVX rats than OVX rats. Similarly, we found lower *Kiss1r* expression in the ARC of OVX rats. In the current study, we focused on diestrus day 1 and proestrus. Analyses of the complete estrous cycle (including estrus and diestrus day2) would be informative considering the change in estrus reported by Adachi et al. (2007). Another PCR-based study by Navarro et al. (2004) showed the estrous cycle dependency of *Kiss1r*

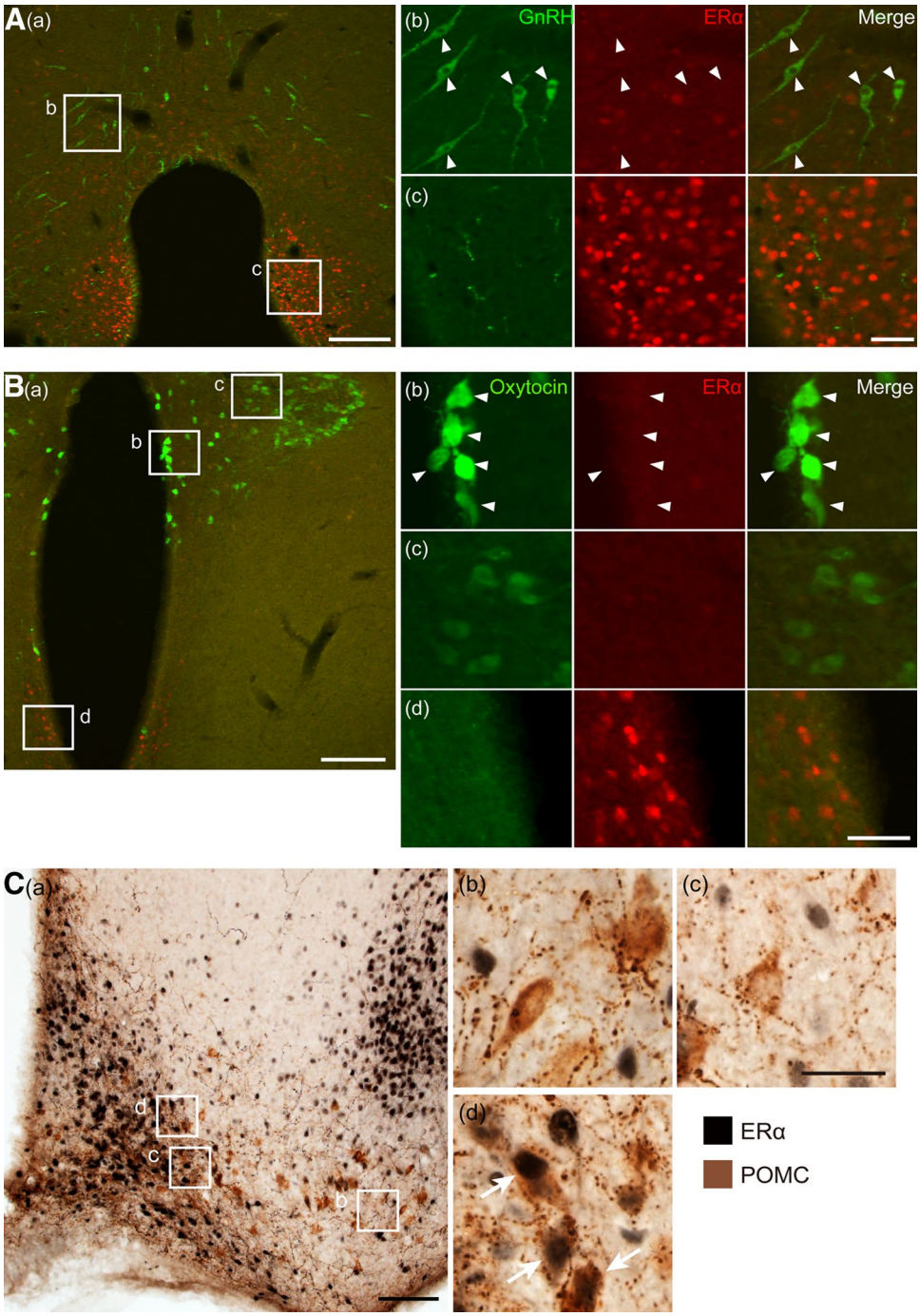

Fig. 3 Colocalization of estrogen receptor alpha (ERα) and gonadotropin-releasing hormone (GnRH), oxytocin, or pro-opiomelanocortin (POMC). Photomicrographs taken from diestrus female rats group are displayed as representative images. **A** Representative images of immunofluorescence (IF) double-labeling for ERα (red) and GnRH (green). a Low-magnification image of the DB. b, c Magnified images of framed area in a. Arrowheads indicate GnRH-immunopositive neurons. Almost no colocalization of GnRH with ERα was observed. Scale bar = 200 µm for a and 50 µm for b, c. **B** Representative images of IF double-labeling for ERα (red) and oxytocin (green). a Low-magnification image of the PVN. b–d Magnified images of framed area in a. Arrowheads indicate oxytocin-immunopositive neurons. Almost no colocalization of oxytocin with ERα was observed. Scale bar = 200 µm for a and 50 µm for b–d. Intensities of ERα signals in each image were adjusted proportionally to the area showing the strongest signal as a reference **A**-c for GnRH, **B**-d for oxytocin neurons. **C** Representative images of immunohistochemical double-labeling for ERα (nickel–cobalt enhanced DAB, black) and POMC (normal DAB, brown). a Low-magnification image of the ARC. b–d Magnified images of framed area in a. Arrows indicate co-expression of ERα and POMC. Scale bar = 100 µm for a and 50 µm for b–d. The co-expression rate was low (approximately 10–15%)

expression in the rat hypothalamus with highest levels at diestrus, which seem to be in conflict with our result showing elevated *Kiss1r* in the PVN in the proestrus. Considering that Navarro's report assessed the *Kiss1r* in the whole hypothalamus, one possible cause for this inconsistency could be the change in *Kiss1r* expression in other *Kiss1r*-expressing hypothalamic regions, e.g., the supraoptic nucleus, premammillary and supramammillary nuclei.

Only a moderate difference in *Kiss1r* mRNA expression was observed in the PVN between diestrus and proestrus, in sharp contrast to the substantial changes observed in kisspeptin expression across the estrous cycle. In our previous study, we reported that a major fraction of *Kiss1r*-expressing neurons in the medial part of the PVN also express oxytocin (Higo et al. 2016), and the current study confirmed the absence of ERα in oxytocin-containing neurons of the PVN. These findings suggest that the change in PVN *Kiss1r* mRNA expression is more likely to be indirectly regulated by estrogen, such as through ER-stimulated kisspeptin release or afferent inputs from ERα-positive interneurons.

Further investigations on these possible indirect pathways are necessary for better understanding of *Kiss1r* regulation in the PVN.

The physiological relevance of the *Kiss1r* change along with estrous cycle in the PVN still remains unknown. However, given the fact that the oxytocin neurons in the PVN receive fibers from kisspeptin neurons in the RP3V in which kisspeptin expression is high at proestrus (Kotani et al. 2001; Herbison 2008; Scott and Brown 2011; Higo et al. 2016), and that oxytocin enhances lordosis behavior in rats (Caldwell et al. 1986), a significant increase in PVN *Kiss1r* expression at proestrus may facilitate female receptive behavior.

High E2 replenishment of OVX rats suppressed *Kiss1r* expression by approximately 60% in the ARC, a region in which most *Kiss1r*-expressing neurons also express POMC. Since colocalization of POMC and ERα was found in less than 15% of ARC neurons, reduced *Kiss1r* expression in the ARC is unlikely due solely to direct ERα-mediated E2 actions. Since POMC neurons make strong reciprocal connection with many forebrain structures, E2 action through other E2-responsive hypothalamic regions is also conceivable (Wang et al. 2015).

In the DB, the neuronal population expressing *Kiss1r* is almost identical to the GnRH neuronal population (Higo et al. 2016). The *Kiss1r* expression in GnRH neurons in the DB is of notable importance considering the indispensability of kisspeptin–KISS1R system for the maintenance of the estrous cycle and fertility (Funes et al. 2003; Seminara et al. 2003). The absence of the change in *Kiss1r* expression in this study indicate the robustness of *Kiss1r* expression in GnRH neurons, and no detectable colocalization of ERα indicates the change in GnRH secretion along with the estrous cycle is regulated via the estrogen feedback mainly on the expression of kisspeptin ligand (García-Galiano et al. 2012) but not on the expression of *Kiss1r*.

Jacobi et al. reported that E2 treatment increased *Kiss1r* expression in the hypothalamic cell line GT1-7, which is often used as a model for GnRH neurons because of similarities in gene expression (Martínez de la Escalera and Clapp 2001; Jacobi et al. 2007; Weiner et al. 1992). However, considering the unchanged DB *Kiss1r* expression in the present study and a report showing ERα expression in GT1-7 cells (Roy et al. 1999), gene regulation may differ between GT1-7 cells and naïve GnRH neurons.

The current study revealed regional differences in the change in *Kiss1r* expression between OVX and gonadally intact females; E2-induced change in the numbers of *Kiss1r*-expressing neurons was observed specifically in the ARC of OVX + high-E2 rats, whereas difference in *Kiss1r*-expression between estrous cycle stages in gonadally intact females was observed specifically in the PVN. Our results also indicate the possible suppressive effect of ovariectomy on *Kiss1r* expression, considering an overall tendency for reduced *Kiss1r* expression in OVX animals in all three analytical regions (DB, PVN, and ARC). These complexities in the change in *Kiss1r* expression may be explained by depletion of other regulatory factors by ovariectomy, such as progesterone, inhibin, and (or) activin. Although OVX and E2 replenishment models are commonly used in reproduction studies, the results of our study underscore the importance of considering non-estrogenic factors affected by OVX.

To date, no study has reported the detailed cellular distribution of *Kiss1r* in male rats. In rats and other mammals, kisspeptin expression shows clear sex differences, with higher expression in females than males in both the RP3V and ARC (Adachi et al. 2007; Kauffman et al. 2007; Takumi et al. 2011). Based on these findings, we speculated that there may be parallel differences in *Kiss1r* expression. However, surprisingly, we found no such sex difference in either loci, at least at the mRNA level. Although the expression of kisspeptin ligand is low in males, previous studies of *Kiss1*- and *Kiss1r*-deficient mice indicate that kisspeptin-KISS1R signaling is indispensable for spermatogenesis and gonadal maturation in males (Mei et al. 2011; Lapatto et al. 2007; Chan et al. 2009). These data indicate that *Kiss1r* expression levels required for spermatogenesis in males are equivalent to that for maintenance of the estrous cycle in females.

In conclusion, the current study demonstrates complex regional differences in *Kiss1r* expression within females. In contrast to kisspeptin, *Kiss1r* expression in the rat brain showed no sex difference in either anatomical distribution or number of expressing cells. Alternatively, there were differences between OVX and E2-supplemented OVX females and between gonadally intact females during diestrus and proestrus in distinct hypothalamic regions (ARC and PVN, respectively). However, these differences appeared independent of ERα suggesting possible indirect regulation of *Kiss1r* expression via E2 actions in other regions and (or) by other factors depleted by ovariectomy. Further research is required to determine how kisspeptin signaling changes during sexual development, reproductive behavior, and the estrous cycle.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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