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ORIGINAL ARTICLE

Role of kisspeptin and Kiss1R in the regulation of prolactin gene expression in rat somatolactotroph GH3 cells

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Abstract

Hypothalamic kisspeptin is a known principal activator of gonadotropin-releasing hormone neurons and governs the hypothalamic-pituitary-gonadal axis. Previous reports have shown that kisspeptin is also released into the hypophyseal portal circulation and directly affects the anterior pituitary. In this study, we examined the direct effect of kisspeptin on pituitary prolactin-producing cells. The rat pituitary somatolactotroph cell line GH3 expresses the kisspeptin receptor (Kiss1R); however, in these cells, kisspeptin failed to stimulate prolactin-promoter activity. When GH3 cells overexpressed Kiss1R, kisspeptin clearly increased prolactin-promoter activity, with a concomitant increase in extracellular signal-regulated kinase (ERK) and cAMP/protein kinase A (PKA) signaling pathways. In the experiments using GH3 cells overexpressing Kiss1R, kisspeptin did not potentiate thyrotropin-releasing hormone (TRH)-induced prolactin-promoter activity, but it potentiated the pituitary adenylate cyclase-activating polypeptide-induced prolactin-promoter activity, with a concomitant enhancement of ERK and PKA signaling pathways. Although the basal and TRH-induced prolactin-promoter activities were not modulated by increasing amounts of Kiss1R expression in GH3 cells, kisspeptin-stimulated prolactin-promoter activity was increased by the amount of Kiss1R overexpression. Endogenous *Kiss1r* mRNA expression in GH3 cells was significantly increased by treatment with estradiol (E2) but not by TRH. In addition, kisspeptin's ability to stimulate prolactin-promoter activity was restored after E2 treatment in non-transfected GH3 cells. Our current observations suggest that kisspeptin might have a direct effect on prolactin expression in the anterior pituitary prolactin-producing cells under the influence of E2, which may regulate Kiss1R expression and function.

Keywords Kisspeptin · Prolactin · Kiss1R · TRH · PACAP

Introduction

Kisspeptin, which is encoded by the *Kiss1* gene, is known for its principal role in reproductive function by regulating the hypothalamic-pituitary-gonadal axis, and it primarily controls gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus [1]. The *Kiss1* gene and the kisspeptin receptor (Kiss1R) are broadly distributed in the brain. In rodents, kisspeptin neurons are located in the two different hypothalamic areas, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC). Kisspeptin neurons in the AVPV region, in which the *Kiss1* gene is upregulated by estradiol (E2), are known to be involved in the E2-induced GnRH/luteinizing hormone (LH) surge, whereas ARC kisspeptin neurons, which coexpress neurokinin B and dynorphin (Dyn) and in which E2 downregulates the *Kiss1* gene, maintain pulsatile release of GnRH [2–5]. In addition to the two major populations, there are other populations of kisspeptin neurons in the hypothalamus, such as those in the ventromedial hypothalamus and paraventricular nucleus [6, 7]. Extrahypothalamic kisspeptin neurons have also been detected in bed nucleus of the stria terminals and median amygdala [6, 8].

Kisspeptin produced by the hypothalamus is known to be released into the peripheral circulation because kisspeptin has been detected in the hypophyseal portal blood [9]. This observation implies that the hypothalamic peptide kisspeptin directly modulates hormone secretion from the anterior pituitary as a hypothalamic factor. In addition,

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kisspeptin and Kiss1R are expressed in peripheral organs outside the central nervous system [10, 11]. The pituitary gland also expresses the *Kiss1* gene and Kiss1R [12], suggesting that pituitary hormones might also be under the influence of kisspeptin in an autocrine and/or paracrine fashion.

The anterior pituitary gland is composed of five major different hormone-secreting cells: corticotrophs, thyrotrophs, gonadotrophs, somatotrophs, and lactotrophs. These cells synthesize and secrete adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), gonadotropins (LH and follicle-stimulating hormone [FSH]), growth hormone (GH), and prolactin under the influence of relatively specific hypothalamic peptides such as corticotropin-releasing hormone, thyrotropin-releasing hormone (TRH), GnRH, and GH-releasing hormone. In addition to the primary secretagogues, several in vitro studies support the hypothesis that hypothalamic kisspeptin can also act at the pituitary level and modulate pituitary function. In rat, bovine, and porcine pituitary cultures, kisspeptin stimulates the release of GH, prolactin, and LH [13–15]. However, the first studies using cultured rat pituitary cells and anterior pituitary fragments did not demonstrate any direct effect on gonadotropin secretion [16, 17]. In addition, in vitro experiments using baboon pituitary cell cultures produced no evidence that ACTH and TSH release are modulated by kisspeptin, although LH and GH release was reported to be stimulated in this culture [18]. As for the gonadotropin regulation by kisspeptin in pituitary gonadotrophs, we have previously reported that kisspeptin had a direct effect on the mouse pituitary gonadotroph cell line LβT2 and increased both LHβ and FSHβ-subunit-promoter transcriptional activities [19].

Previous studies suggest that kisspeptin might have a direct effect on pituitary prolactin-producing cells. Kisspeptin increases the prolactin release from cultured bovine anterior pituitary cells, but its effect was less potent than that of TRH [20]. However, in another study using rat anterior pituitary cell cultures, kisspeptin failed to modulate prolactin release [21]. In addition, kisspeptin has been shown to stimulate prolactin secretion and gene expression by directly acting at the pituitary level in goldfish;[22] however, the capacity of this peptide to modulate prolactin has not been confirmed in in vivo studies using goats [23].

In this study, we focused on the direct effect of kisspeptin on prolactin-producing pituitary cells. We utilized GH3 cells, which are a clonal strain of rat pituitary tumor and can synthesize and secrete both prolactin and GH [24]. We confirmed the presence of Kiss1R in these cells, and examined the direct effect of kisspeptin in these cells.

Materials and methods

Materials

The following chemicals and reagents were sourced as follows: fetal bovine serum (FBS) and trypsin (GIBCO, Invitrogen, Carlsbad, CA); Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, TRH (Sigma-Aldrich, St. Louis, MO); pituitary adenylate cyclase-activating polypeptide 38 (PACAP38, Peptide Institute, Osaka, Japan); kisspeptin-10 (KP10) (ANA SPEC, Fremont, CA); serum response element (SRE) and cAMP-response element (CRE) firefly luciferase reporter genes (pSRE-Luc and pCRE-Luc) and pCI-neo (Promega, Madison, WI).

Cell culture

GH3 cells were plated in 35-mm tissue culture dishes and incubated in high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 h, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin and incubated without (control) or with test reagents for the indicated times.

Western blot analysis

GH3 cell extracts were lysed on ice with RIPA buffer (phosphate-buffered saline [PBS], 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 0.1 mg/mL phenylmethylsulfonyl fluoride, 30 mg/mL aprotinin, and 1 mM sodium orthovanadate, scraped for 20 s, and centrifuged at 14,000×*g* for 10 min at 4 °C. Protein concentration in the cell lysate supernatants was measured using the Bradford method. Denatured protein (10 µg per well) was resolved in 10% SDS polyacrylamide gel electrophoresis gels according to standard protocols. Protein was transferred onto polyvinylidene difluoride membranes (Hybond-P PVDF, Amersham Biosciences, Little Chalfont, UK), which were blocked for 2 h at room temperature in Blotto (5% milk in Tris-buffered saline). Membranes were incubated with anti-Kiss1R antibody (1:200 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX) in Blotto overnight at 4 °C and washed 3 times for 10 min per wash with Tris-buffered saline/1% Tween. Subsequent incubation with horseradish peroxidase-conjugated monoclonal antibody was performed for 1 h at room temperature in Blotto, and additional washes were performed appropriately. Following enhanced chemiluminescence detection (Amersham Biosciences), membranes were exposed to X-ray film (Fujifilm, Tokyo, Japan). Extracts from rat anterior pituitary tissue

were used as positive control, whereas extracts from COS7 cells, which are devoid of Kiss1R, were used as negative control [25].

Receptor overexpression

The human GPR54 (Kiss1R) vector was generously provided by Dr. Ursula Kaiser (Brigham and Women's Hospital and Harvard Medical School, Boston, MA) and the PACAP type I receptor (PAC1R)-expressing vector (HA-tagged PAC1R/pEF-BOS in pCAM17) was kindly provided by Prof. A. Baba (Osaka University) [26]. Cells were transiently transfected via electroporation with either Kiss1R or PAC1R expression vectors. An empty vector (pCI neo) served as the mock control.

Transfections and luciferase assays

The prolactin promoter reporter construct used was generated by fusing −609/+12 of the prolactin gene to the firefly luciferase cDNA in pGL3 (PRL-Luc), as previously described [27]. To determine the extracellular signal-related kinase (ERK) and cAMP/protein kinase A (PKA)-mediated signaling activity, pSRE-Luc (2.0 µg/well; contains tandem repeats of the *Sre* enhancer [×4] upstream of the firefly luciferase gene) and pCRE-Luc (2.0 µg/well; contains tandem repeats of the CRE enhancer [×4] upstream of the firefly luciferase gene) were applied. GH3 cells were transiently transfected by electroporation [28] with 2.0 µg/dish of reporter construct and 0.1 µg of pRL-TK (Promega), which expresses *Renilla* luciferase, and plated in 35-mm tissue culture dishes. When Kiss1R and PAC1R were expressed in GH3 cells, Kiss1R- and PAC1R-expressing vectors were transiently transfected together with these luciferase expression vectors. After stimulation, cells were washed with ice-cold PBS and lysed with Passive Lysis Buffer (Promega). Cell debris was pelleted by centrifugation at 14,000×*g* for 10 min at 4 °C, and firefly luciferase and *Renilla* luciferase activities were measured in the supernatants with the Dual-Luciferase Reporter Assay System using a luminometer (TD-20/20) (Promega) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity to correct for transfection efficiency, and the results are expressed as the fold increase compared to the unstimulated control. All experiments were performed independently, three times, each with triplicate samples.

RNA preparation, reverse transcription, RT-PCR, and real-time quantitative RT-PCR

Total RNA from untreated or treated GH3 cells was extracted using commercially available TRIzol-S (GIBCO BRL Life Technologies) according to the manufacturer's instructions. Total RNA of female rat anterior pituitary tissue, which was excised under deep sodium pentobarbital anesthesia, was used as positive control. This protocol was approved by the committee of the Experimental Animal Center for Integrated Research in Shimane University (IZ27-82). To obtain cDNA, 1.0 µg of total RNA was reverse transcribed using an oligo-dT primer (Promega), and was prepared using a First-Strand cDNA Synthesis Kit (GIBCO, Invitrogen) in reverse transcription (RT) buffer. The preparation was supplemented with 10 mM dithiothreitol, 1 mM each dNTP, and 200 units of RNAse inhibitor/human placenta ribonuclease inhibitor (Ribonuclease Inhibitor, Code No. 2310, Takara, Tokyo, Japan) in a final volume of 10 µl. The reaction was incubated at 37 °C for 60 min. For the detection of *Kiss1r* mRNA, after PCR amplification using primers for *Kiss1r* (sense: 5′-CTGCCACAGACGTCACTTTC-3′, antisense: 5′-ACATACCAGCGGTCCACACT-3′) [29], amplicons were electrophoresed in a 2.0% agarose gel and visualized with ethidium bromide staining. cDNAs from rat anterior pituitary tissues and COS7 cells were used as positive and negative controls, respectively. Quantification of *Kiss1r* and *Prl* mRNA was obtained through real-time quantitative PCR (ABI Prism 7000, Perkin Elmer Applied Biosystems, Foster City, CA) following the manufacturer's protocol (User Bulletin No. 2), and utilizing Universal ProbeLibrary Probes and FastStart Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for *Kiss1r* [29] and *Prl* (sense: 5′-AATGACGGAAATAGATGATTG-3′, antisense: 5′-CCAGTTATTAGTTGAAAVAGA-3′) [27], the simultaneous measurement of the mRNA of interest and GAPDH mRNA permitted normalization of the amount of cDNA added per sample. For each set of primers, a no-template control was included. The thermal cycling conditions were 95 °C for 10 min for denaturation, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The cycle threshold (Ct) was determined using PRISM 7000 software and post-amplification data were analyzed using the delta-delta Ct method with Microsoft Excel.

Statistical analysis

All experiments were independently repeated at least three times. Each experiment in each experimental group was performed using either triplicate samples (luciferase assay) or duplicate samples (real-time RT-PCR). Briefly, when we determined the mRNA expression, two samples were assayed in duplicate. Six averages from three independent experiments were statistically analyzed. For the luciferase assay, three samples were assayed in one experiment, and three averages were statistically analyzed. Data are expressed as the mean ±

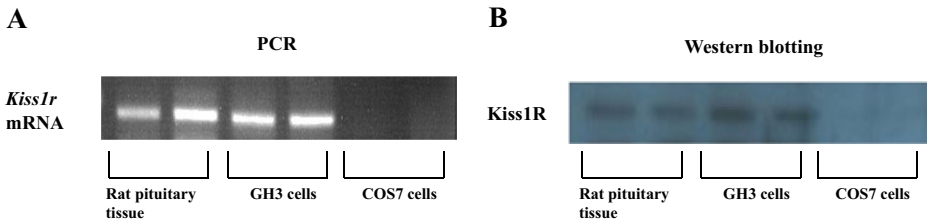

Fig. 1 Expression of Kiss1R in GH3 cells. **a** Total RNA from GH3 cells and rat anterior pituitary tissues were prepared and RT-PCR was carried out for 40 cycles using *Kiss1r*-specific primers. PCR products were resolved in a 2.0% agarose gel and visualized with ethidium bromide staining. **b** Cell lysates (10 µg) from GH3 cells and rat anterior pituitary tissues were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibody against Kiss1R. The bands were visualized using horseradish peroxidase-conjugated secondary antibody. Tissues from rat anterior pituitary and extracts from COS7 cells were used as positive and negative controls, respectively

standard error of the mean. Statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test. $P < 0.05$ was considered statistically significant.

Results

Expression of Kiss1R and the effect of kisspeptin on prolactin-promoter activity in GH3 cells

First, we examined whether GH3 cells express Kiss1R. RT-PCR analysis using specific primers for *Kiss1r* revealed that *Kiss1r* mRNA could be detected in the extracts from rat anterior pituitary tissues as well as GH3 cells (Fig. 1a). *Kiss1r* mRNA was not detected in COS7 cells, which are a fibroblast-like cell line derived from monkey kidney. Western blotting analysis using anti-Kiss1R antibody revealed that Kiss1R protein was also expressed in GH3 cells (Fig. 1b). Next, we examined the direct effect of kisspeptin on prolactin expression using GH3 cells. Stimulating the GH3 cells with increasing concentrations of kisspeptin failed to modulate the transcriptional activity of the prolactin promoter. TRH, a known prolactin secretagogue, stimulated the prolactin promoters 3.02 ± 0.18-fold ($P < 0.01$) in these cells (Fig. 2a). Because endogenous Kiss1R did not respond to exogenous kisspeptin, we overexpressed Kiss1R in the GH3 cells. When GH3 cells were transfected with Kiss1R-expressing vectors, the cells clearly responded to kisspeptin and increased the activity of the prolactin promoter. In Kiss1R-overexpressing GH3 cells, kisspeptin stimulation significantly increased prolactin-promoter activity compared to the untreated cells: 1.69 ± 0.09-fold ($P < 0.05$) at 10 nM and 2.49 ± 0.11-fold ($P < 0.01$) at 1 µM kisspeptin (Fig. 2b).

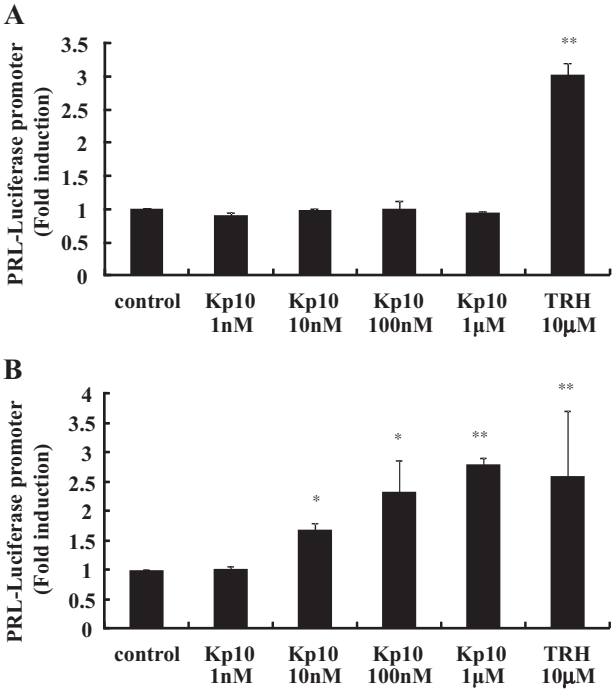

Fig. 2 Effect of kisspeptin on the activity of the prolactin (PRL) promoter. GH3 cells were transfected without (mock) **a** or with 2.0 µg of Kiss1R-expressing vector **b**, together with pRL-TK (0.1 µg) plus 2.0 µg of PRL-Luc vector. At 48 h after transfection, cells were treated with increasing doses of kisspeptin (Kp10) for 6 h. A firefly luciferase assay was performed to examine prolactin-promoter activity, which was normalized to *Renilla* luciferase activity, and is expressed as the fold induction over the unstimulated controls. Data are expressed as the mean ± standard error of the mean (three independent experiments were performed using triplicate samples). $*P < 0.05$; $**P < 0.01$ vs. control

Effect of kisspeptin on SRE and CRE-promoter activity in GH3 cells overexpressing Kiss1R

To examine the signaling pathways activated by kisspeptin in Kiss1R-overexpressing GH3 cells, we performed SRE- and CRE-luciferase promoter assays. SRE is a DNA domain in the promoter region that binds to ERK-mediated transcription factors, and SRE-promoter activity reflects ERK-mediated signaling pathway activity. The CRE promoter is a known target of the CRE-binding protein, and the CRE-luciferase reporter system reflects the activity of the cAMP/PKA signaling pathway. In mock-transfected GH3 cells, neither the SRE nor the CRE promoter was activated by

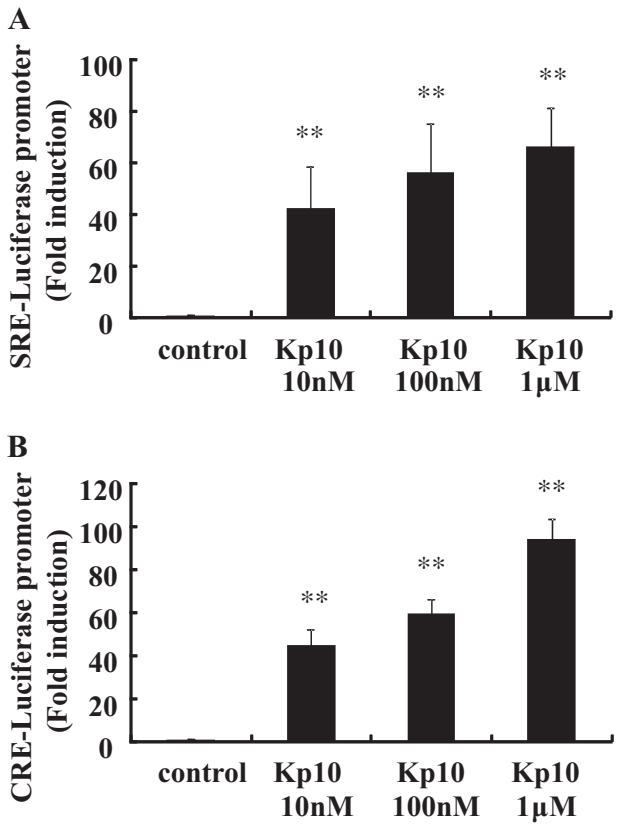

Fig. 3 Effect of kisspeptin on SRE- and CRE-promoter activities. GH3 cells were transfected with 2.0 μg of Kiss1R-expressing vector, together with pRL-TK (0.1 μg) and 2.0 μg of SRE-Luc **a** or CRE-Luc **b** vector. Forty-eight hours after transfection, cells were treated with increasing amounts of kisspeptin (Kp10) for 6 h. A firefly luciferase assay was performed to examine SRE and CRE-promoter activity, which was normalized to *Renilla* luciferase activity and is expressed as the fold induction over the unstimulated controls. Data are expressed as the mean ± standard error of the mean (three independent experiments performed using triplicate samples). $^{**}P < 0.01$ vs. control

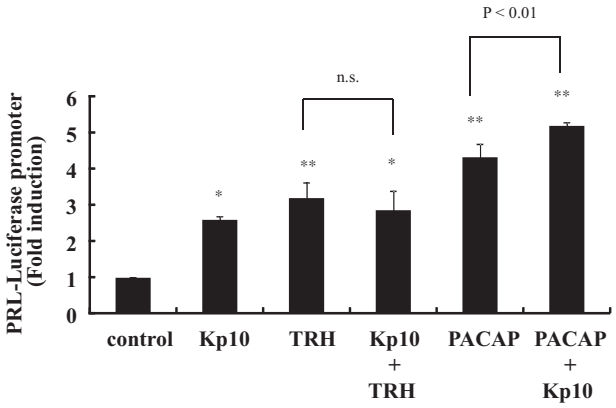

Fig. 4 Effect of kisspeptin, TRH, and PACAP on prolactin-promoter activity. GH3 cells were transfected with 2.0 μg of Kiss1R-expressing and 2.0 μg of PAC1R-expressing vectors, together with 2.0 μg of PRL-Luc and pRL-TK (0.1 μg) vectors. Forty-eight hours after transfection, cells were treated with 100 nM kisspeptin (Kp10), 100 nM TRH, 100 nM PACAP, or both Kp10 and TRH or Kp10 and PACAP for 6 h. A firefly luciferase assay was performed to examine prolactin-promoter activity, which was normalized to *Renilla* luciferase activity and is expressed as the fold induction over the unstimulated controls. Data are expressed as the mean ± standard error of the mean (three independent experiments performed using triplicate samples). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control. The difference between PACAP and PACAP + Kp10 treatment was statistically significant ($P < 0.01$). n.s., difference was not statistically significant

kisspeptin stimulation (data not shown). When Kiss1R was overexpressed in these cells, both SRE- and CRE- promoters were dramatically activated by kisspeptin. At 1 μM kisspeptin stimulation, SRE- and CRE- promoters were activated 66.46 ± 15.19-fold and 94.42 ± 9.62-fold, respectively, relative to the control (Fig. 3a, b).

Effect of kisspeptin on TRH or PACAP-induced prolactin-promoter activity

TRH is a principal secretagogue for prolactin. In addition, PACAP participates in prolactin regulation [30]. To clearly observe the effect of both kisspeptin and PACAP, both receptors were overexpressed for the experiments. In GH3 cells overexpressing both Kiss1R and PAC1R, kisspeptin and TRH similarly stimulated prolactin-promoter activity by 2.60 ± 0.10-fold and 3.18 ± 0.45-fold, respectively, compared with the unstimulated controls, but combined treatment with kisspeptin and TRH did not enhance their individual effects (TRH alone, 3.18 ± 0.45-fold vs. kisspeptin + TRH, 2.87 ± 0.53-fold; not significant). However, PACAP stimulated prolactin-promoter activity to a greater degree compared to that stimulated by kisspeptin (4.32 ± 0.39-fold), and combined stimulation with kisspeptin and PACAP significantly further increased prolactin-promoter activity compared with that by kisspeptin or PACAP alone (PACAP alone, 4.32 ± 0.39-fold vs. kisspeptin + PACAP, 5.20 ± 0.11-fold; $P < 0.01$) (Fig. 4). Next, we determined the SRE and CRE-promoter activities stimulated by kisspeptin, TRH, and PACAP. Kisspeptin dramatically increased SRE-promoter activity (99.79 ± 10.27-fold) compared with that by TRH stimulation (6.44 ± 0.70-fold) in Kiss1R- and PAC1R-overexpressing GH3 cells. Combined stimulation with kisspeptin and TRH failed to potentiate the effect of kisspeptin alone (105.47 ± 19.62-fold). PACAP stimulated SRE-promoter activity to a lesser degree than that stimulated by kisspeptin (14.88 ± 1.30-fold), and combined stimulation with kisspeptin and PACAP significantly increased the SRE-promoter activity compared with that stimulated by kisspeptin alone (kisspeptin alone, 99.79 ± 10.27-fold vs. kisspeptin + PACAP, 150.91 ± 23.71-fold; $P < 0.05$) (Fig. 5a). The patterns of CRE-promoter activity stimulation were distinct from those of the SRE promoter. Both kisspeptin and TRH significantly increased CRE-promoter activity 26.73 ± 0.91-fold and 71.69 ± 4.24-fold,

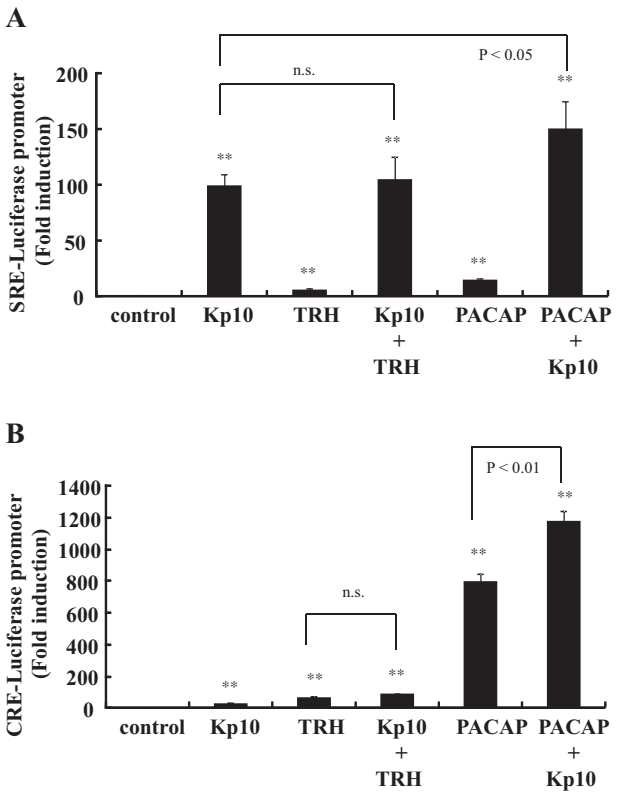

Fig. 5 Effect of kisspeptin, TRH, and PACAP on SRE- and CRE-promoter activities. GH3 cells were transfected with 2.0 µg of Kiss1R-expressing and 2.0 µg of PAC1R-expressing vectors, together with pRL-TK (0.1 µg) and 2.0 µg of SRE-Luc **a** or CRE-Luc **b** vectors. Forty-eight hours after transfection, cells were treated with 100 nM kisspeptin (Kp10), 100 nM TRH, 100 nM PACAP, or both Kp10 and TRH or Kp10 and PACAP for 6 h. A firefly luciferase assay was performed to examine SRE and CRE-promoter activity, which was normalized to *Renilla* luciferase activity and is expressed as the fold induction over the unstimulated controls. Data are expressed as the mean ± standard error of the mean (three independent experiments performed using triplicate samples). $^{**}P < 0.01$ vs. control. The difference in SRE-promoter activity between Kp10 and PACAP + Kp10 treatment was statistically significant ($P < 0.05$). The difference in CRE-promoter activity between PACAP and PACAP + Kp10 treatment was statistically significant ($P < 0.01$). n.s., difference was not statistically significant

respectively, and combined stimulation with kisspeptin and TRH did not enhance the effect of TRH alone (TRH alone, 71.69 ± 4.24-fold vs. kisspeptin + TRH, 89.66 ± 2.60-fold; not significant). PACAP more potently activated CRE-promoter activity, 798.28 ± 50.06-fold, compared with kisspeptin or TRH. Although TRH-induced CRE-promoter activity was not modified in the presence of kisspeptin, PACAP-stimulated CRE-promoter activity was significantly potentiated in the presence of kisspeptin (PACAP alone, 798.28 ± 50.06-fold vs. kisspeptin + PACAP, 1182.67 ± 58.97-fold; $P < 0.01$) (Fig. 5b).

Effect of increasing amounts of Kiss1R-expressing vector transfection in GH3 cells on kisspeptin and TRH-induced prolactin-promoter activity

Next, we examined how the cell responses changed according to Kiss1R expression levels. GH3 cells were transfected with different amounts of Kiss1R expression vector and stimulated with kisspeptin. The basal activity and TRH or kisspeptin-induced fold induction of prolactin-promoter activity were compared. Basal activity of the prolactin promoter was unchanged by transfection of increasing amounts of Kiss1R expression vector (Fig. 6a). TRH-induced fold induction of prolactin-promoter activity was not modified by the dose of transfected Kiss1R expression vector (Fig. 6b). However, kisspeptin-stimulated prolactin-promoter activity was significantly higher in the cells transfected with 2.0 and 4.0 µg of Kiss1R vector (3.75 ± 1.27-fold and 2.44 ± 0.58-fold, respectively) compared with that in cells transfected with 1.0 µg of Kiss1R vector (1.66 ± 0.25-fold) (Fig. 6c).

Effect of E2 on Kiss1R expression and function

Next, we examined how endogenous *Kiss1r* mRNA is regulated in GH3 cells. TRH (100 nM) did not stimulate *Kiss1r* gene expression in GH3 cells. However, treatment of cells with 100 nM E2 significantly increased *Kiss1r* mRNA expression, which was increased 1.72 ± 0.2-fold compared with untreated cells (Fig. 7a). *Kiss1r* mRNA was not increased by concentrations of E2 lower than 100 nM (data not shown). Furthermore, we found that GH3 cells acquired responsiveness to kisspeptin after the 48-h treatment with 100 nM E2. GH3 cells that were not overexpressing Kiss1R were treated with E2 for 48 h and then stimulated with kisspeptin. In E2-treated GH3 cells, but not in untreated GH3 cells, kisspeptin significantly increased *Prl* mRNA expression 1.90 ± 0.16-fold compared to unstimulated cells (Fig. 7b).

Discussion

The importance of hypothalamic kisspeptin in the regulation of hypothalamic GnRH neurons has been well documented, but accumulating evidence suggests that kisspeptin also plays a role as a hypophysiotropic hormone and acts directly within the pituitary gland. As for prolactin control by kisspeptin at the pituitary level, previous in vivo studies showed divergent responses. Central intracerebroventricular injection of kisspeptin reduced the prolactin release in both male and female mice [31], while Szawka et al. observed an increase in prolactin by the same kisspeptin stimulation [21]. Stimulatory effects of kisspeptin on prolactin

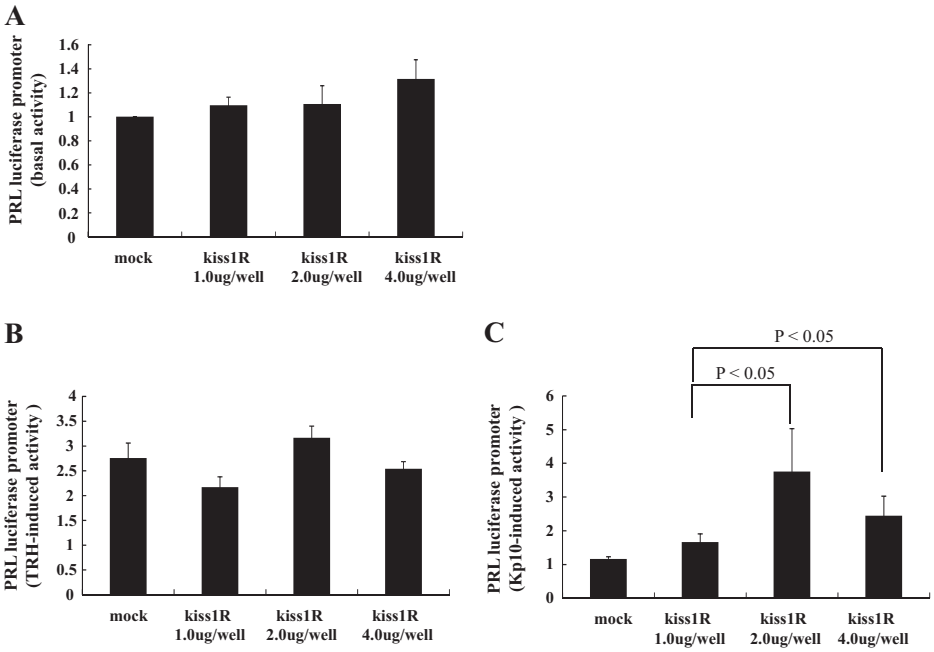

Fig. 6 Effects of Kiss1R overexpression on the basal levels and the kisspeptin- and TRH-induced fold induction of prolactin-promoter activity. GH3 cells were transfected with 1.0–4.0 µg of Kiss1R-expressing vector together with 2.0 µg of PRL-Luc and pRL-TK (0.1 µg) vectors. Forty-eight hours after transfection, cells were treated with 100 nM TRH **b** and 100 nM kisspeptin (Kp10) **c** for 6 h. A firefly luciferase assay was performed to examine prolactin (PRL) promoter activity, which was normalized to *Renilla* luciferase activity and expressed as basal **a** and the fold induction over unstimulated controls in the mock-transfected group. The fold induction of TRH-stimulated **b** and Kp10-stimulated **c** cells over unstimulated cells was calculated. Data are expressed as the mean ± standard error of the mean (three independent experiments performed using triplicate samples). $^{**}P < 0.01$ vs. mock control. The differences between the 1.0 µg and higher amounts of Kiss1R-expressing cells in Kp10-induced prolactin-promoter activity were statistically significant ($P < 0.05$)

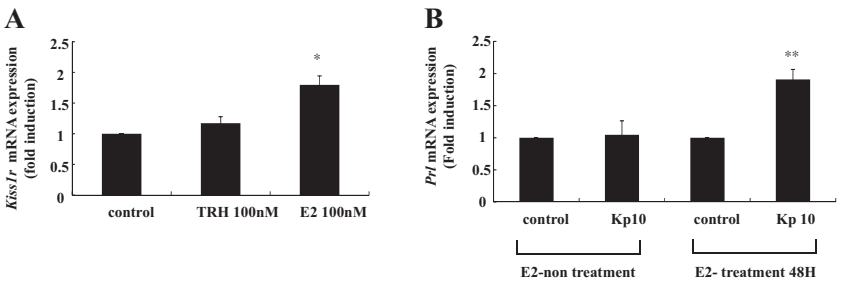

Fig. 7 Effects of estradiol on *Kiss1r* mRNA expression and receptor function. **a** GH3 cells were treated with 100 nM TRH and 100 nM estradiol (E2) for 48 h. *Kiss1r* mRNA levels were measured by quantitative real-time PCR after mRNA extraction and reverse transcription. **b** GH3 cells were pre-treated in the presence or absence of 100 nM E2 for 48 h, and then stimulated with kisspeptin (Kp10) for an additional 48 h. Prolactin (*Prl*) mRNA levels were measured by quantitative real-time PCR after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and normalized to GAPDH mRNA levels. Results are expressed as the fold induction over unstimulated cells and presented as the mean ± standard error of the mean of three independent experiments, each performed with duplicate samples. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control

expression and release are also observed in the goldfish pituitary [22], but not in goats [23]. It was also reported that kisspeptin could inhibit dopamine neurons in the hypothalamus and modulate prolactin output [21].

In this study, we sought to clarify the action of kisspeptin at the single population of prolactin-producing lactotrophs to evaluate the direct effect of kisspeptin on the anterior pituitary cells. Because of the difficulty of isolating single-cell populations of pituitary lactotrophs from anterior pituitary cells, we used the rat somatolactotroph cell line, GH3. These cells are a clonal strain of rat pituitary tumor and can synthesize and secrete both prolactin and GH [24]. GH3 cells respond to TRH and increase their synthesis and secretion of prolactin, but TRH reduces the synthesis of GH

[24, 27]. We found that GH3 cells express Kiss1R. Because GH3 cells are a pituitary prolactin-producing cell model, it is plausible that normal prolactin-producing cells in the pituitary gland express Kiss1R and are directly influenced by hypothalamic kisspeptin. Unexpectedly, GH3 cells did not respond to kisspeptin and failed to modulate the transcriptional activity of the prolactin promoter. We sometimes encounter similar problems when we use immortalized-cell models. The pituitary gonadotroph cell line LβT2 expresses Kiss1R, but these cells do not respond to kisspeptin without Kiss1R overexpression [19]. Similarly, the mouse GnRH-producing cell model GT1-7 expresses Kiss1R, but kisspeptin failed to induce responses in these cells without Kiss1R overexpression [32]. We postulate that endogenous Kiss1R is reduced or not functional, probably due to cell immortalization or multiple passages in these immortalized-cell models. Thus, we used GH3 cells overexpressing Kiss1R as a prolactin-producing cell model in our experiments to determine the effect of kisspeptin.

When GH3 cells overexpressed Kiss1R, they clearly responded to kisspeptin and increased prolactin-promoter activity. These observations clearly demonstrated that the kisspeptin/Kiss1R system in prolactin-producing cells has the ability to stimulate prolactin expression. Both SRE- and CRE-reporter luciferase activities were increased by kisspeptin stimulation in GH3 cells overexpressing Kiss1R, suggesting that the overexpressed Kiss1R coupled with Gq and Gs proteins and increased both ERK and cAMP/PKA signaling pathways. Previous studies revealed that kisspeptin can activate a variety of signals via Kiss1R, which includes Gq protein-involved activation of phospholipase C (PLC) and subsequent accumulation of inositol triphosphate (IP3), intracellular Ca^{2+} mobilization, and activation of protein kinase C. Kisspeptin also activates ERK, P38 MAPK, and PI3K/Akt [33]. Although early studies showed that Kiss1R does not couple with Gs protein and does not increase cAMP accumulation [11, 34], it was subsequently shown that kisspeptin can increase cAMP accumulation in goldfish pituitary cells [35] and in GnRH-producing GT1-7 cells [32].

Being the principal prolactin secretagogue, TRH could of course stimulate prolactin-promoter activity in our experiments. The hypothalamic peptide PACAP also works as a prolactin-stimulating factor [36]. Interestingly, combined stimulation with kisspeptin and TRH failed to potentiate their individual effects on the prolactin promoter; however, the combination of kisspeptin and PACAP further stimulated prolactin-promoter activity compared to that stimulated individually. The TRH receptor couples with Gq protein and activates PLC-mediated signaling pathways, which includes IP3 accumulation or Ca^{2+} mobilization [37]. PACAP receptors, such as the PACAP type I receptor, mainly couple with Gs protein, which binds to adenylate cyclase, leading to the accumulation of cAMP and subsequent activation of PKA;[38] PACAP has also been shown to activate ERK signaling pathways in a PKA-dependent manner [28]. We presume that combined treatment with kisspeptin and TRH did not enhance their prolactin-producing ability because Kiss1R and the TRH receptor share common signaling pathways that are mainly initiated by Gq protein and PLC. Indeed, stimulation of SRE- and CRE-promoter activities was not potentiated by the combined treatment with kisspeptin and TRH. In contrast, prolactin-promoter activity could be enhanced by combined stimulation with kisspeptin and PACAP, with concomitant enhancement of SRE- and CRE-promoter activities, hypothetically because the main signal transduction systems are distinct between Kiss1R and the PACAP receptor.

GH3 cells express Kiss1R, but they did not respond to kisspeptin. Interestingly, the function of endogenous Kiss1R in these cells was recovered in the presence of 100 nM E2. In addition, we found that the same concentration of E2 could increase the expression of Kiss1R in these cells. Although it is still unclear whether our experiments actually reflect the physiological situation, our current observations imply that E2 has some roles in prolactin-producing cells by modulating the expression and function of Kiss1R. The importance of E2 in the functional effects of kisspeptin was previously described. In ewes, primary pituitary cell cultures responded to kisspeptin and increased LH secretion only when the cells were obtained during the follicular phase of the estrous cycle, while no response was seen in cells from the luteal phase or from ovariectomized animals [9]. In an ovariectomized rat model, pre-exposure to E2 was effective in achieving maximal LH release in response to kisspeptin [39]. Similarly, the effect of kisspeptin on gonadotropin release in women is greater in the preovulatory phase but lower in the follicular phase of the menstrual cycle [40]. In addition, an in vitro study using GnRH-producing GT1-7 cells demonstrated that E2 induced Kiss1R expression [41].

The prolactin-inducing ability of kisspeptin was altered by the amount of Kiss1R expression vector transfected into the GH3 cells. In short, kisspeptin-stimulated prolactin-promoter activity increased with increasing amounts of Kiss1R vector. A similar phenomenon was also observed in gonadotropin-producing cells. The mouse gonadotroph cell line LβT2 expresses Kiss1R and increasing Kiss1R expression potentiates the ability of kisspeptin to increase LHβ-subunit promoter transcriptional activity [19]. Although the amount of Kiss1R vector did not modify the basal and TRH-induced transcriptional capacities of the prolactin promoter in this study using GH3 cells, the basal activity of both LHβ- and FSHβ-subunit promoters in LβT2 cells was modified [19]. The increase in Kiss1R number

under the influence of E2 might introduce some other influences on prolactin-producing cells.

Regarding the inconsistencies between previous reports on the action of kisspeptin and our current observations of the effect of E2 on kisspeptin/Kiss1R functions, it is plausible that conflicting results concerning the direct effect of kisspeptin on prolactin secretion and gene expression depend on the hormonal milieu of the experimental models. Developmental stage, male versus female, or day of the estrous cycle of the experimental female animals might determine the sensitivity of pituitary lactotrophs to kisspeptin in the in vivo studies. Furthermore, kisspeptin may not only directly affect lactotrophs, but may also influence other regulators of prolactin. Indeed, it was reported that dopamine neurons, known negative regulators of prolactin, receive synaptic input from kisspeptin neurons and modulate prolactin secretion [21, 42, 43]. Furthermore, a recent study using a female rat model demonstrated that kisspeptin stimulation of prolactin release requires estrogen receptor α [44]. On the other hand, experiments using pituitary cell cultures were also influenced by many other local factors within the anterior pituitary because primary-culture cells contained multiple different cell types including at least five different hormone-secreting cells. In addition, as with the in vivo studies, the characteristics of pituitary cells would be dissimilar depending on the species, developmental stage, sex difference, or the day of the estrous cycle when the cells were obtained. The culturing periods prior to using the cells might also influence the responsiveness of the cells to kisspeptin because the disappearance of E2 after removing pituitaries from the animals could diminish the function of Kiss1R in the pituitary cells.

In this study, we used somatolactotroph GH3 cells overexpressing Kiss1R to examine the direct effect of kisspeptin on prolactin-producing cells. We found that Kiss1R is expressed in a pituitary prolactin-producing cell model and obtained evidence that kisspeptin has a direct effect on this cell by stimulating prolactin production. Furthermore, we showed that E2 plays an important role in modulating kisspeptin's effect on these cells. We realize that our current study and results do not completely reflect the physiological condition of prolactin-producing cells in vivo. However, these prolactin-producing cells originating from rat express Kiss1R, suggesting that normal lactotrophs in the pituitary gland express Kiss1R. In addition, from the observations that Kiss1R was functional in GH3 cells (by artificial Kiss1R overexpression or E2 treatment) and that kisspeptin could stimulate intracellular signaling and stimulate prolactin gene expression, we could speculate that normal lactotrophs, which express functional Kiss1R, would respond to kisspeptin and increase prolactin production. In our current study using clonal prolactin-producing cells, the cells were not influenced by any other factors except kisspeptin when we stimulated them with kisspeptin. Our current observations suggest an important role of kisspeptin/Kiss1R in the regulation of pituitary lactotroph functions.

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Compliance with ethical standards

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

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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