Kisspeptin Induces Expression of Gonadotropin-releasing Hormone Receptor in GnRH-producing GT1-7 Cells Overexpressing G protein-coupled Receptor 54

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Running title: GnRHR expression by kisspeptin in GPR54-expressing GT1-7 cells

Funding: This work was supported by a Grant in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (H.K).

Declaration of interest: There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Abstract

Kisspeptin signaling through its receptor is crucial for many reproductive functions. However, the molecular mechanisms and biomedical significance of the regulation of GnRH neurons by kisspeptin have not been adequately elucidated. In the present study, we found that kisspeptin increases GnRH receptor (GnRHR) expression in a GnRH-producing cell line (GT1-7). Because cellular activity of G protein-coupled receptor 54 (GPR54) and GnRHR was limited in GT1-7 cells, we overexpressed these receptors to clarify receptor function. Using luciferase reporter constructs, the activity of both the serum response element (Sre) promoter, a target for extracellular signal-regulated kinase (ERK), and the cyclic AMP (cAMP) response element (Cre) promoter were increased by kisspeptin. Although GnRH increased Sre promoter activity, the Cre promoter was not significantly activated by GnRH. Kisspeptin, but not GnRH, increased cAMP accumulation in these cells. Kisspeptin also increased the transcriptional activity of GnRHR; however, the effect of GnRH on the GnRHR promoter was limited and not significant. Transfection of GT1-7 cells with constitutively active MEK kinase (MEKK) and protein kinase A (PKA) increased GnRHR expression. In addition, GnRHR expression was further increased by co-overexpression of MEKK and PKA. The Cre promoter, but not the Sre promoter, was also further activated by co-overexpression of MEKK and PKA. GnRH significantly increased the activity of the GnRHR promoter in the presence of cAMP. The present findings suggest that kisspeptin is a potent stimulator of GnRHR expression in GnRH-producing neurons in association with ERK and the cAMP/PKA pathways.

1. Introduction

Gonadotropin-releasing hormone (GnRH), a hypothalamic decapeptide, is released into the hypophyseal portal vascular system in a pulsatile manner, where it functions as a principle regulator of the reproductive system by stimulating the synthesis and release of pituitary gonadotropins. Although GnRH has been defined as the final neuronal component of the hypothalamus-pituitary-gonadal axis, kisspeptin has recently been identified as playing an essential role in the regulation of GnRH neurons [13, 26, 31]. Kisspeptin, known previously as metastin, was originally identified as a product of the metastasis suppressor gene, *KiSS-1*, and is a natural ligand of G protein-coupled receptor 54 (GPR54; also known as KiSS1R) [22]. In 2003, inactivating mutations in GPR54 were found in patients with idiopathic hypogonadotropic hypogonadism, which is characterized by low levels of sex steroids and gonadotropin [5, 27]. Mutations in human *KiSS-1* and *KiSS-1R*, which encode GPR54, were found in patients with central precocious puberty [28, 30], and kisspeptin- and GPR54-knockout mice were shown to exhibit infertility [4, 18]. Thus, kisspeptin is currently recognized as the most potent activator of the hypothalamus-pituitary-gonadal axis.

GnRH mediates the intermittent release of the pituitary gonadotropins—luteinizing hormone (LH) and follicle-stimulating hormone (FSH)—in a pulsatile manner [32]. The cellular mechanism of action of kisspeptin was investigated using immortalized GnRH neurons. It has been suggested that GPR54 activates the Gq protein/phospholipase C/inositol (1,4,5)-triphosphate (IP3) pathway, resulting in the release of calcium from calcium storage sites [15, 16]. On the other hand, studies have revealed that kisspeptin does not modify basal or forskolin-induced cyclic AMP (cAMP) levels in HEK293 and CHO-K1 cells overexpressed with GPR54 [15, 19]. Protein

kinase C activation, as well as activation of the extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3 kinase (PI3K)/Akt pathways, was also observed in a human papillary thyroid cancer cell model overexpressing GPR54 [29].

The GnRH receptor (GnRHR) is expressed in the plasma membrane of gonadotrophs of the anterior gland, which are themselves regulated by GnRH. High- and low-frequency GnRH pulse stimulation produces distinctive patterns of GnRHR expression levels in gonadotrophs. GnRHR is increased at higher GnRH pulse frequencies at which expression of the LHβ subunit gene is optimally stimulated, whereas expression of the FSHβ gene is favored by a lower density of receptors and a lower frequency of GnRH pulse stimulation [1, 10], suggesting the involvement of GnRHR expression levels in the differential regulation of gene expression of the gonadotropin LHβ and FSHβ subunits. Because it was difficult to culture hypothalamic neuronal cells and to isolate GnRH neuronal cells, we used GT1-7 cells as a model for hypothalamic GnRH neurons, as these cells are widely used as *in vitro* models of GnRH-producing neurons [17]. Kisspeptin elevates intracellular calcium levels, while the PKC signaling pathway downstream of GPR54 has been shown to mediate GnRH expression and secretion from GT1-7 cells [23]. It has also been demonstrated that the kisspeptin/GPR54 system plays a significant role in the positive regulation of GnRH release and expression in GT1-7 cells [21], which also express GnRHR and respond to GnRH [20]. However, the means by which GnRHR is regulated in GnRH neuronal cells have remained unknown. The hormone-producing function of gonadotrophs is mediated by the expression patterns of gonadotrophic GnRHR. Therefore, it is possible that GnRHR expression within the GnRH neuron is self-regulated, which ultimately modulates reproductive function. In this study, we used GT1-7 cells to elucidate the

effect of kisspeptin and GnRH on GnRH-producing neurons.

2. Materials and Methods

2.1 Materials

The following chemicals and reagents were obtained from the indicated sources: Fetal bovine serum (FBS) and trypsin (GIBCO, Invitrogen, Carlsbad, CA); Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, GnRH, 8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate (CPT-cAMP) (Sigma Chemical Co., St. Louis, MO); Kisspeptin (ANA SPEC, Fremont, CA); Phosphorylated ERK (anti-P-ERK) antibody, anti-ERK antibody, and suitable horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA); Serum response element (Sre) and cAMP-response element (Cre) firefly luciferase reporter gene (pSre-Luc and pSre-Luc), pFC-MEKK and pFC-PKA (Stratagene, La Jolla, CA); and pCI-neo (Promega, Madison, WI).

2.2 Cell culture

GT1-7 cells [17], kindly provided by Dr. P. Mellon of the University of California (San Diego, CA), were plated in 35-mm tissue culture dishes and incubated with high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C under 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 the test reagents for the indicated periods.

2.3 Reporter Plasmid Construct and Luciferase Assay

Wild type human GnRH receptor (hGnRHR) rxpression vector, construction of

the human GPR54 (KISS1R) vector, and the reporter construct of -1164/+62 of mouse GnRHR gene (GnRHR-Luc) to the firefly luciferase cDNA were generously provided by Dr. Ursula Kaiser (Bigham and Women's Hospital and Harvard University, MA). Cells were transiently transfected by electroporation with either 2.0 μg/well of hGnRHR or GPR54 expression vectors. An empty vector (pCI- neo) served as a mock control. When the activity of the promoter containing Sre and Cre was measured, the cells were transfected with either pSre-Luc (2.0 μg DNA) or pCre-Luc (2.0 μg DNA), which contain five-tandem repeats of the Sre or Cre enhancer (×4), respectively, upstream of the firefly luciferase gene, with or without (mock) receptor overexpression. After incubation with test reagents, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with passive lysis buffer (Promega). After centrifugation at 15,000 ×*g* at 4 °C, firefly luciferase and *Renilla* luciferase activity in the supernatant was measured using a luminometer (TD-20/20) and the Dual-Luciferase Reporter Assay System (both Promega) according to the manufacturer's protocol. Luciferase activity was normalized to *Renilla* luciferase activity to correct for transfection efficiency, and the results were expressed as fold increases compared with the unstimulated control.

2.4 Western blot analysis

GT1-7 cells were rinsed with PBS then lysed on ice with RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% 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. The protein concentration was measured in the cell lysates using the Bradford method of protein quantitation. Denatured protein per well (10 μg) was separated on a 10% SDS-PAGE

gel 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 phospho-ERK antibody (p-ERK) (1:250 dilution; Santa Cruz Biotechnology, Inc.) in Blotto overnight at 4 °C and washed 3 times for 10 min per wash with Tris-buffered saline/1% Tween. Subsequent incubation with monoclonal HRP-conjugated antibody was carried out for 1 h at room temperature in Blotto, and appropriate additional washes were performed. Following chemiluminescence (ECL) detection (Amersham Biosciences), membranes were exposed onto X-ray film (Fujifilm, Tokyo, Japan). After strip washing (Restore buffer, Pierce Chemical Co., Rockford, IL), membranes were reprobed with ERK antibody (T-ERK) (1:10000 dilution; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, followed by incubation with HRP-conjugated secondary antibody and continuation of the procedure as described above. Films were analyzed by densitometry, and the intensity of P-ERK was normalized to that of T- ERK to correct protein loading. The corrected results for ERK phosphorylation were expressed as fold induction over the controls.

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

Total RNA from treated and untreated GT1-7 cells was extracted using commercially available Trizol-S (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. To obtain cDNA, 1.0 μg of total RNA was reverse transcribed using an oligo-dT primer (Promega, Madison, WI), and was prepared using a First Strand cDNA Synthesis Kit (Invitrogen Life Technologies) in

reverse transcription (RT) buffer. The preparation was supplemented with 0.01 dithiothreitol, 1 mM of each dNTP, and 200 units of RNAse inhibitor/human placenta 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. Quantification of GnRHR was obtained through real-time quantitative polymerase chain reaction (PCR; ABI Prism 7000, Perkin Elmer Applied Biosystems, Foster City, CA) following the manufacturer's protocol (User Bulletin No. 2) and utilizing a Universal Probe Library Probe and Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for GnRHR [11], the simultaneous measurement of mRNA and GAPDH permitted normalization of the amount of cDNA added per sample. For each set of primers, a no template control was included. Thermal cycling conditions were as follows: 10 min denaturation at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The crossing threshold was determined using PRISM 7000 software, and post amplification data were analyzed using the delta-delta CT method in Microsoft Excel.

2.6 Measurement of cAMP accumulation

Cells overexpressing GPR54 or GnRHR were plated in 96-well plates at a density of 10^5 cells/well and cultured for 48 h. Cells were then stimulated with kisspeptin or GnRH in serum-free DMEM medium for 1 h. Intracellular cAMP levels were measured using the direct cAMP ELISA kit (Enzo Life Science, Inc, Farmingdale, NY)

2.7 Statistical analysis

All experiments were repeated independently at least three times. Each

experiment in each experimental group was performed using triplicate samples (luciferase assays) or duplicate samples (Western blot). Data are expressed as mean ± SEM values. Statistical analysis was performed using the one-way ANOVA followed by Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Activation of Sre and Cre promoters by kisspeptin and GnRH in GT1-7 cells

We examined the effect of kisspeptin and GnRH on Sre- and Cre-luciferase promoters in GT1-7 cells. Sre is a DNA domain in the promoter region that binds to ERK-mediated transcription factors, and activation of Sre promoters reflects the activity of ERK-mediated signaling pathways. On the other hand, 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 pathway. In mock-transfected GT1-7 cells, both kisspeptin and GnRH failed to stimulate either Sre or Cre promoter activity. As there is the possibility that GT1-7 cells did not respond to kisspeptin or GnRH because of the reduced number of GPR54 and GnRHR in these cells, we transfected these receptors to GT1-7 cells to determine the effect of kisspeptin and GnRH. After overexpression of GPR54 and GnRHR, both kisspeptin and GnRH increased Sre promoter activity by 18.58 ± 4.39-fold and 5.40 ± 1.73-fold, respectively (Fig. 1A and B). Although Cre promoter activity was significantly activated by 2.40 ± 0.12-fold by kisspeptin, it was not significantly increased by GnRH (1.18 ± 0.14-fold) (Fig. 1C and D).

3.2 ERK activation and intracellular cAMP accumulation by kisspeptin and GnRH

We examined whether ERK was activated and whether the intracellular cAMP level was increased by kisspeptin and GnRH in these cells. In GT1-7 cells transfected with GPR54 or GnRHR expression vectors, both kisspeptin and GnRH increased ERK phosphorylation (Fig. 2A and C). On the other hand, kisspeptin, but not GnRH, increased the accumulation of intracellular cAMP in these cells (Fig. 2B and D).

3.3 Effect of kisspeptin and GnRH on GnRHR transcriptional activity

Next, we examined the effect of kisspeptin and GnRH on GnRHR promoter activity. In mock-transfected GT1-7 cells, kisspeptin failed to stimulate GnRHR promoter activity, while in the cells with GPR54 overexpression, kisspeptin significantly increased GnRHR promoter activity by 1.97 ± 0.02-fold (Fig. 3A). On the other hand, the effect of GnRH on GnRHR promoters was limited and not significant, even when GnRHR was overexpressed; it showed an increase of only 1.34 ± 0.13-fold in response to GnRH stimulation (Fig. 3B). To examine the specificity of kisspeptin's effect on GnRH promoters in GnRH neuronal cells, GPR54 was overexpressed in pituitary gonadotroph LβT2 cells and stimulated. In LβT2 cells overexpressed with GPR54, kisspeptin increased Cre promoter activity (Fig. 4A); however, kisspeptin failed to increase GnRHR promoter activity (Fig. 4B).

3.4 Effect of overexpression of pFC-MEKK and pFC-PKA on GT1-7 cells

It was speculated that kisspeptin increased GnRHR promoter activity in association with both the ERK and cAMP pathways because GnRH failed to produce a significant effect on GnRHR promoters in the absence of cAMP/PKA/Cre activation. Next, we examined the direct effect of the ERK and cAMP/PKA pathways on GnRHR promoters by transfecting GT1-7 cells with pFC-MEKK and pFC-PKA. These vectors induced expression of constitutively active MEKK (which ultimately stimulates ERK) and PKA. Both pFC-MEKK and pFC-PKA specifically increased the Sre and Cre promoters, respectively (Fig. 5A and B). Notably, combined expression of MEKK and PKA further increased Cre promoter activity, although MEKK had no such effect (Fig. 5B). On the other hand, PKA expression did not modify the effect of MEKK on Sre

promoters (Fig. 5A). Individual overexpression of MEKK and PKA increased GnRHR promoter activity, while combined overexpression of MEKK and PKA further increased GnRHR promoter activity compared with individual overexpression of MEKK and PKA (Fig. 5C). mRNA expression of GnRHR mirrored these effects. Similarly, GT1-7 cells transfected with pFC-MEKK or pFC-PKA significantly increased mRNA expression of GnRHR. Furthermore, co-transfection of the cells with pFC-MEKK and pFC-PKA further increased GnRHR mRNA expression compared with co-transfection with pFC-MEKK or pFC-PKA (Fig. 5D).

3.5 Effect of cAMP on GnRH-stimulated GnRHR promoter activity

The effect of cAMP GnRH-stimulated GnRHR promoter activity was examined. In this series of experiments, GnRH slightly but significantly increased GnRHR promoter activity. Preincubation of cells with the cAMP analogue, CPT-cAMP, further and significantly increased GnRHR promoter activity by GnRH stimulation compared to that without cAMP (Fig. 6).

4. Discussion

We examined the regulation of GnRHR by kisspeptin and GnRH because GnRHR expression plays a pivotal role in neuroendocrine cells. For example, GnRH regulates the expression of its receptor in anterior pituitary gonadotrophic cells. GnRH also stimulates GnRHR expression in gonadotrophs, and pulsatile GnRH alters the expression of GnRHR according to the GnRH pulse frequency interval, which ultimately controls the differential expression of LH and FSH [1, 10]. Similarly, the receptor for pituitary adenylate cyclase-activating polypeptide (PACAP) within gonadotrophic cells is regulated by its ligand and mediates the differential expression of the gonadotropin subunit [11, 12]. Thus, cell surface receptor expression in GnRH-producing cells might potentiate their function, which might ultimately affect reproductive function.

First, we examined how GT1-7 cells respond to kisspeptin. To our surprise, reporter gene assays demonstrated that neither Sre- nor Cre-luciferase promoters were increased by kisspeptin and GnRH. Because a number of studies have shown that GT1-7 cells respond to kisspeptin and GnRH [20, 21, 23], we suspect that the expression of GPR54 and GnRHR in the present study might have been reduced by changes in cell character due to multiple passages. Thus, we overexpressed these receptors in GT1-7 cells and analyzed the results. Before this, however, we confirmed that the mRNA for endogenous GPR54 was indeed expressed in our GT1-7 cells prior to overexpression and that GPR54 mRNA was significantly increased after GPR54 overexpression. In addition, we confirmed that our GT1-7 cells expressed GnRH mRNA. As expected, after the transfection of GPR54- or GnRHR-expressing vectors, GT1-7 cells clearly responded and increased Sre-luciferase activity by kisspeptin and

GnRH. This activity suggests that GPR54 and GnRHR have the ability to stimulate intracellular signal transduction when abundantly expressed.

Both kisspeptin and GnRH increased Sre promoter activity. However, The Cre promoter, which reflects activation of the cAMP/PKA pathways, was increased by kisspeptin but not by GnRH. Indeed, intracellular cAMP levels were increased by kisspeptin, but not by GnRH. Previous studies on the characterization of GPR54 in heterologous cell models expressing GPR54 have involved the analysis of intracellular signaling pathways activated by this receptor. Kisspeptin can activate a variety of signals via GPR54, which includes Gq-protein-coupled cascades, such as activation of PLC and subsequent accumulation of IP3, intracellular Ca^{2+} mobilization, and activation of PKC. Kisspeptin also activates ERK, P38MAPK, and PI3K/Akt [2]. On the other hand, it has been shown that GPR54 does not couple with Gs or Gi subfamilies [19] and does not promote cAMP accumulation in response to kisspeptin [15]. In the present study, cAMP was increased and the Cre promoter was activated by kisspeptin in GT1-7 cells overexpressing GPR54. These findings suggest that GPR54 has the ability to stimulate the cAMP/PKA pathways. Artificial expression of GPR54 might activate the cAMP/PKA pathways. However, since GnRHR, a Gq-protein-coupled receptor increased neither cAMP accumulation nor Cre promoter activity, even when overexpressed artificially in GT1-7 cells, GPR54 might be capable of coupling to Gs protein in GT1-7 cells. In other words, this receptor might exist as a Gs-protein-coupled receptor in GnRH-producing neurons.

Kisspeptin mediates the increase of GnRHR in GnRH-producing neuronal cells overexpressing GPR54. However, because kisspeptin increases cAMP accumulation with a subsequent increase in Cre promoters, activation of the cAMP/PKA pathway

might be necessary to induce GnRHR expression in GnRH-producing neuronal cells. To exclude the possibility that exogenous induction of GPR54 artificially increases GnRHR expression, we overexpressed GPR54 in pituitary gonadotrophic LβT2 cells. LβT2 cells with overexpressed GPR54 significantly increased Cre promoter activity via kisspeptin stimulation, but no GnRHR transcriptional activity was induced by kisspeptin. These findings indicate that exogenous GPR54 transmits the kisspeptin signal to the cell, while activated intracellular signaling molecules do not stimulate gene expression of GnRHR in non GnRH-producing cells. Thus, we concluded that the activation of GnRHR promoters by kisspeptin is an event specific to GnRH-producing neuronal cells.

It was speculated that both the cAMP/PKA and ERK pathways are necessary for inducing GnRHR expression because kisspeptin increases both Cre and Sre promoter activity. However, GnRH in the present study activates only Sre promoters. In experiments involving pFC-MEKK and pFC-PKA, both of which overexpress constitutively active MEKK and PKA, overexpression of MEKK and PKA increased GnRHR transcriptional activity as well as mRNA expression, respectively. Some studies have reported crosstalk between the ERK and PKA pathways by demonstrating the activation of Cre promoters by an ERK-mediated protein kinase, probably through $p70^{S6K}$ [24, 33]. However, we detected no such evidence because GT1-7 cell transfected by MEKK and PKA specifically activated Sre and Cre promoters. Both the ERK and PKA pathways might have the ability to individually stimulate GnRHR expression in these cells. Interestingly, co-transfection of GT1-7 cells with MEKK and PKA further stimulated GnRHR promoters compared with transfection of only one. The potentiated patterns of GnRHR promoter activity by combined overexpression of MEKK and PKA were quite similar to those of Cre promoters. These observations suggest the importance

of the cAMP/PKA pathways in the induction of GnRHR. GnRHR transcription was regulated by several ubiquitous factors such as cAMP response element binding protein (CREB) and activator protein 1 (AP1) as well as tissue-specific combination including steroidogenic factor 1 (SF1) and LIM homeodomain proteins ISL-1 and LHX3 [8]. The PROP-1/OTX1 and NUR77 response elements are also identified in the promoter region of GnRHR [25]. Although we did not examine the effect of the ERK and cAMP/PKA pathways on the transcriptional mechanisms by kisspeptin and GnRH activity, all or some of these transcription factors are supposedly mediated by kisspeptin-induced signal-transduction systems. CREB is inherently activated by the cAMP/PKA pathways. In addition, AP1 was identified as the target of the ERK pathway in several studies [14, 34], while SF1 has been reported to be regulated by cAMP-dependent signaling [3, 6] and ERK signaling [7, 9].

Taken together, we speculate that although both ERK and PKA are necessary to induce GnRHR expression, ERK activation plays a more pivotal role in maintaining the effect of kisspeptin on GnRHR promoters. On the other hand, some activation of the cAMP/PKA pathways by kisspeptin might be necessary to induce GnRHR expression in tandem with ERK activation by kisspeptin. The observation that GnRH, which did not induce a significant increase in GnRHR promoter activity, further stimulated the GnRHR promoter in the presence of cAMP supports this hypothesis.

The present study showed that kisspeptin, but not GnRH, stimulates GnRHR expression in GnRH-producing GT1-7 cells. Because we investigated the effects of these peptides on GnRHR expression using GT1-7 cells artificially overexpressing GPR54 or GnRHR, it is uncertain whether this phenomenon occurs *in vivo*. In addition, the present findings should be considered in light of a possible alteration of the

characteristics of GT1-7 cells—including GPR54 and GnRH receptor numbers—due to multiple passages. On the other hand, all reported phenomena were observed within GnRH neuronal cells. Thus, it remains unclear whether or not kisspeptin-induced GnRHR expression within GnRH-producing neurons potentiates the function of gonadotrophs. Nevertheless, the alteration of GnRHR number within GnRH neurons might regulate GnRH production and ultimately affect reproductive functions.

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Figure legends

Fig. 1

Effect of kisspeptin and GnRH on Sre and Cre promoter activity

GT1-7 cells were transfected without (Mock) and with 2.0 μg of GPR54 (A, C) or a GnRHR-expressing vector (B, D), together with both 2.0 μg of Sre-Luc (A, B) or Cre-Luc (C, D) and PRL-TK (0.1 μg) vectors. At 48 h after transfection, cells were treated with 100 nM kisspeptin (Kp-10) or 100 nM GnRH for 6 h. A luciferase assay was performed to examine Sre and Cre promoter activity, which was normalized to PRL-TL activity, and is expressed as the fold difference of activation over the unstimulated controls. Data are expressed as mean ± SEM values (three independent experiments were performed using triplicate samples). **P < 0.01 vs. control.

Fig. 2

Effect of kisspeptin and GnRH on ERK phosphorylation and intracellular cAMP accumulation

GT1-7 cells were transfected without (Mock) and with 2.0 μg of GPR54 (A, B) or GnRHR-expressing vector (C, D). (A, C) At 48 h after transfection, cells were treated with 100 nM kisspeptin (Kp-10) or 100 nM GnRH. Then, 10 min after stimulation, cells were harvested, and cell lysates (10 μg) were subjected to SDS-PAGE followed by Western blotting and incubation with antibody against phosphorylated ERK (P-ERK) and total ERK (T-ERK). The visualized bands show scanning densitometry using NIH Image and normalized to total ERK. (B, D) Transfected GT1-7 cells were plated in 96-well plates and incubated for 60 min, and intracellular cAMP concentration was measured as described in materials and methods. **P < 0.01 vs. control.

Fig. 3

Effect of kisspeptin and GnRH on GnRHR promoter activity

GT1-7 cells were transfected without (Mock) and with 2.0 μg of GPR54 (A) or GnRHR-expressing vector (B), together with PRL-TK (0.1 μg) vectors. At 48 h after transfection, cells were treated with 100 nM of kisspeptin (Kp-10) or 100 nM of GnRH for 6 h. A luciferase assay was performed to examine GnRHR promoter activity, which was normalized to PRL-TL activity, and is expressed as the fold difference of activation over the unstimulated controls. Data are expressed as mean ± SEM values (three independent experiments were performed using triplicate samples). **P < 0.01 vs. control.

Fig. 4

Effect of kisspeptin on GnRHR promoter activity in GPR54 overexpressed LβT2 cells

LβT2 cells were transfected without (Mock) and with 2.0 μg of GPR54, together with both 2.0 μg of Cre-Luc (A) or GnRHR-Luc (B) and PRL-TK (0.1 μg) vectors. At 48 h after transfection, cells were treated with 100 nM of kisspeptin (Kp-10) for 6 h. A luciferase assay was performed to examine Cre and GnRHR promoter activity, which were normalized to PRL-TL activity, and is expressed as the fold difference of activation over the unstimulated controls. Data are expressed as mean ± SEM values (three independent experiments were performed using triplicate samples). **P < 0.01 vs. control.

Fig. 5

Effect of overexpression of pFC-MEKK and pFC-MEKK on Sre and Cre promoter activity and GnRHR expression

GT1-7 cells were transfected with 0.1 μg PRL-TK vector and 2.0 μg of Sre- (A), Cre- (B), and GnRHR-luciferase vectors (C) together with 2.0 μg pFC-MEKK or pFC-PKA, or both. After 48 h of culture, luciferase activity was measured, and activity was expressed as the fold difference of stimulation over control promoter activity. (D) GT1-7 cells were transfected with 2.0 μg pFC-MEKK or pFC-PKA or both. After 24 h, real-time PCR was performed using GnRHR-specific primers after mRNA extraction. As a Mock control, 2.0 μg of PCI-neo vector was transfected. Data are expressed as mean ± SEM values (three independent experiments were performed using triplicate samples). ** $P < 0.01$, *$P < 0.05$ vs. mock control. The differences between PKA and PKA+MEKK on Cre and GnRHR promoter activity were statistically significant ($P < 0.01$ and $P < 0.05$). The differences between MEKK and MEKK+PKA and between PKA and PKA+MEKK were also statistically significant ($P <0.05$). n.s.: Difference was not statistically significant.

Fig. 6

Effect of cAMP analogue on GnRH-stimulated GnRHR promoter activity

GT1-7 cells were transfected with 2.0 μg of GnRHR-expressing vector, together with 2.0 μg of GnRHR- luciferase vectors and PRL-TK (0.1 μg). At 48 h after transfection, cells were preincubated with 100 mM of CPTcAMP for 60 min and further treated without or with 100 nM of GnRH for 6 h. A luciferase assay was performed to examine GnRHR promoter activity, which was normalized to PRL-TL activity. Results are

expressed as the fold difference of activation over the unstimulated controls. Data are expressed as mean ± SEM values (three independent experiments were performed using triplicate samples). ** $P < 0.01$, * $P < 0.05$ vs. control. The difference between GnRH and GnRH+cAMP was statistically significant ($P< 0.05$).

Fig 1

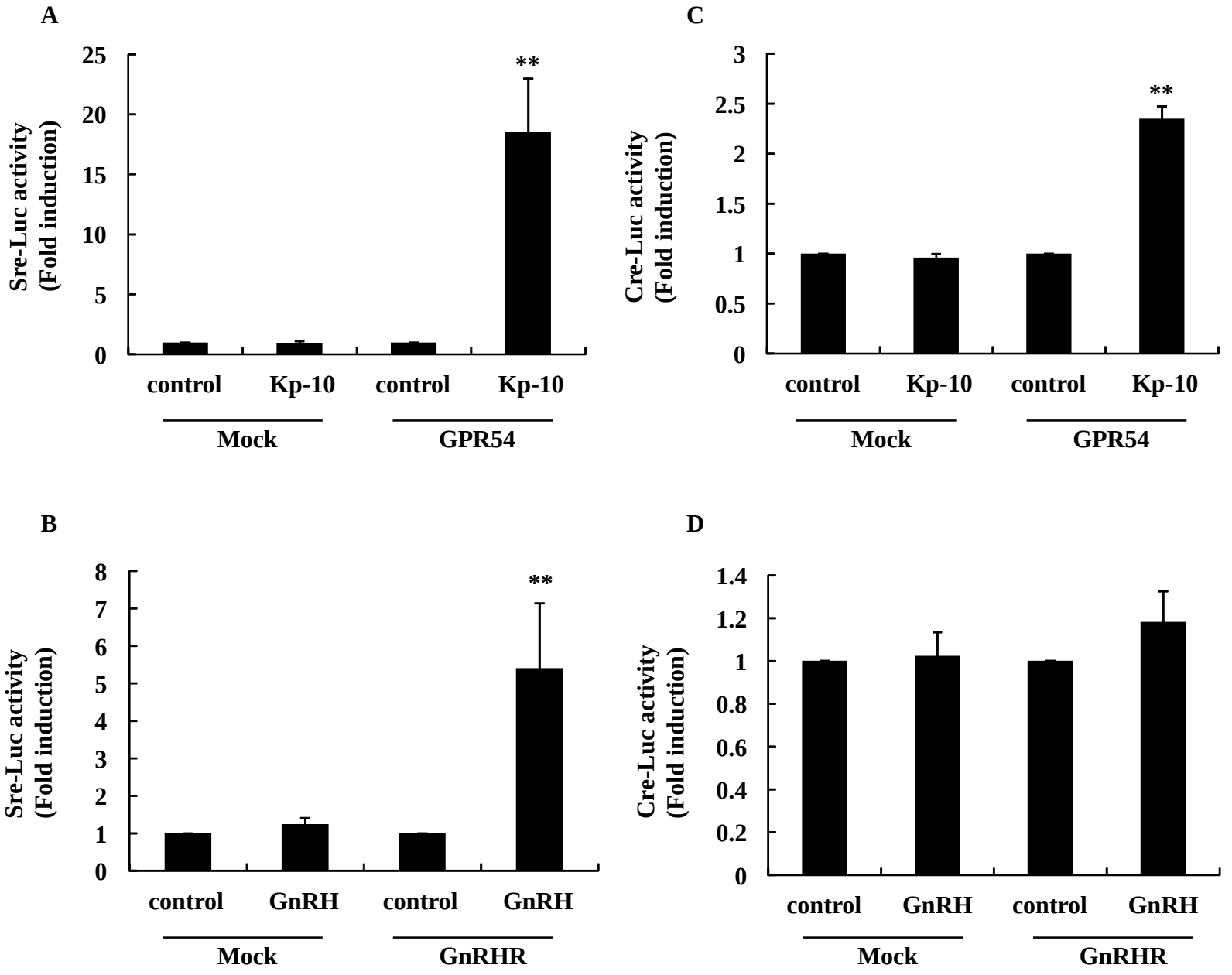

A
Sre-Luc activity
(Fold induction)
25
20
15
10
5
0
**
control
Kp-10
control
Kp-10
Mock
GPR54
C
Cre-Luc activity
(Fold induction)
3
2.5
2
1.5
1
0.5
0
**
control
Kp-10
control
Kp-10
Mock
GPR54
B
Sre-Luc activity
(Fold induction)
8
7
6
5
4
3
2
1
0
**
control
GnRH
control
GnRH
Mock
GnRHR
D
Cre-Luc activity
(Fold induction)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
GnRH
control
GnRH
Mock
GnRHR

Fig 2

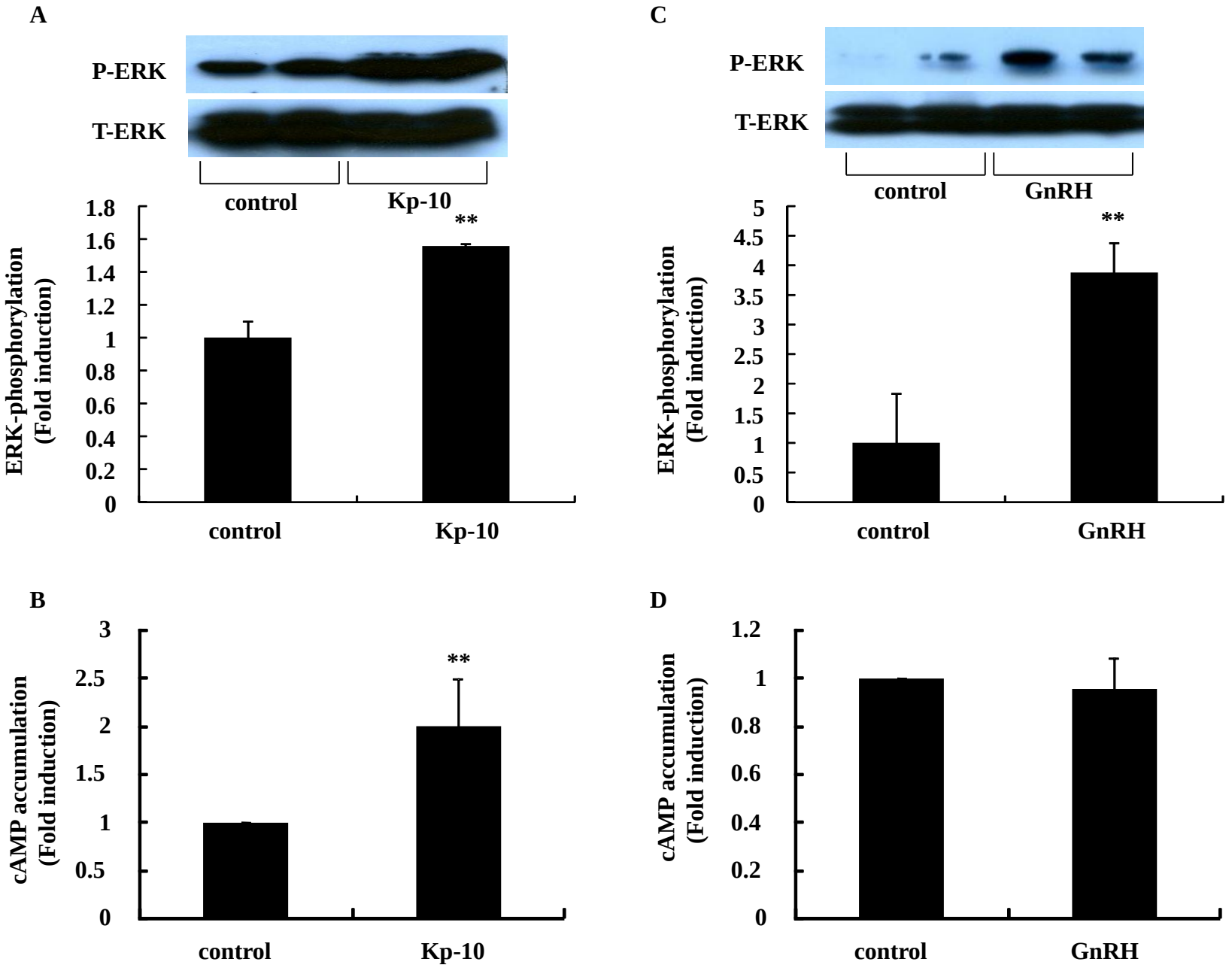

A
P-ERK
T-ERK
control
Kp-10
ERK-phosphorylation (Fold induction)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
control
Kp-10
C
P-ERK
T-ERK
control
GnRH
ERK-phosphorylation (Fold induction)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
control
GnRH
B
cAMP accumulation (Fold induction)
3
2.5
2
1.5
1
0.5
0
**
control
Kp-10
D
cAMP accumulation (Fold induction)
1.2
1
0.8
0.6
0.4
0.2
0
control
GnRH

Fig 3

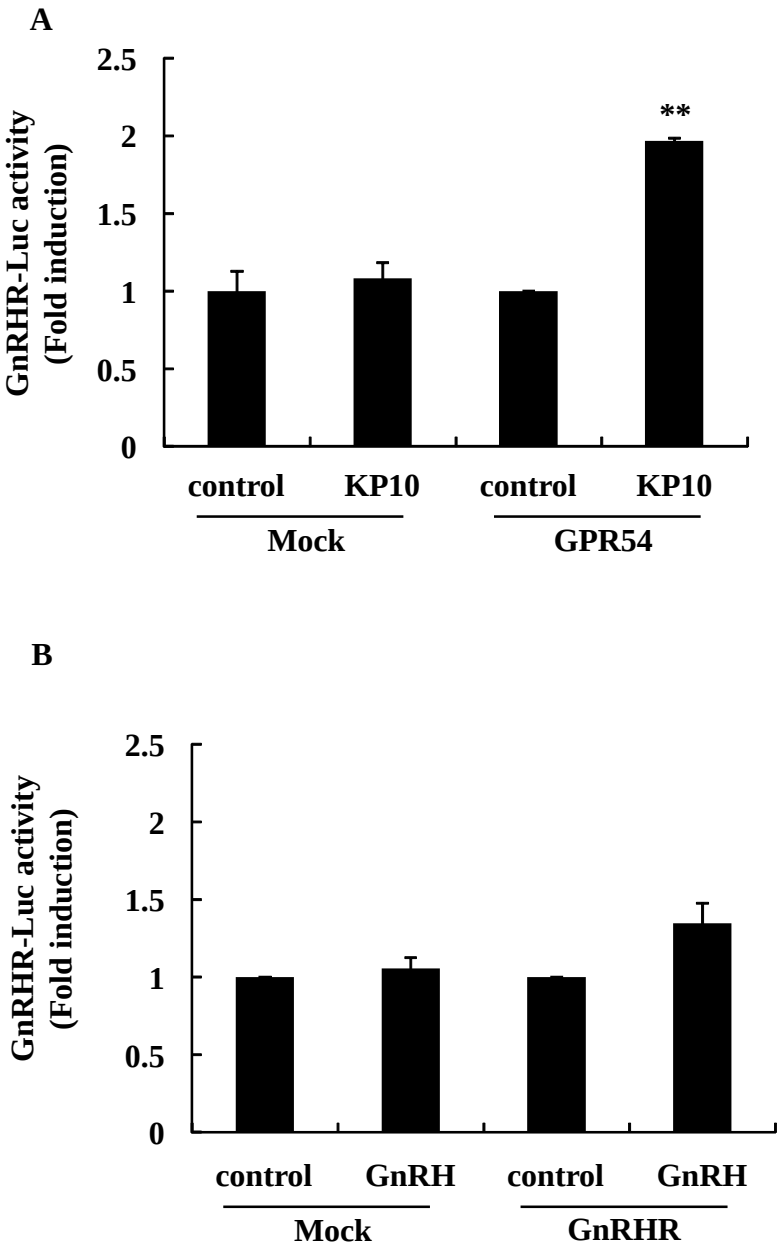

A
GnRHR-Luc activity
(Fold induction)
2.5
2
1.5
1
0.5
0
**
control
KP10
control
KP10
Mock
GPR54
B
GnRHR-Luc activity
(Fold induction)
2.5
2
1.5
1
0.5
0
control
GnRH
control
GnRH
Mock
GnRHR

Fig 4

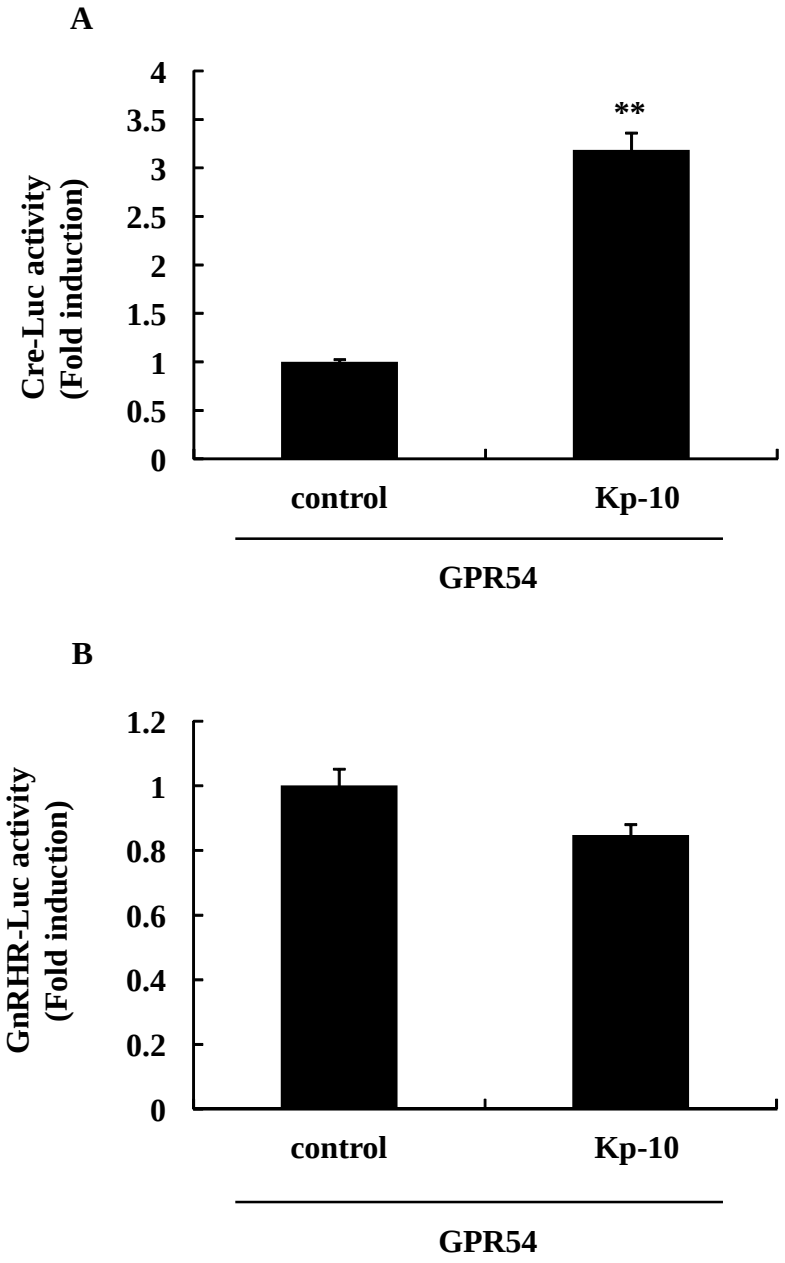

A
Cre-Luc activity
(Fold induction)
4
3.5
3
2.5
2
1.5
1
0.5
0
**
control
Kp-10
GPR54
B
GnRHR-Luc activity
(Fold induction)
1.2
1
0.8
0.6
0.4
0.2
0
control
Kp-10
GPR54

Fig 5

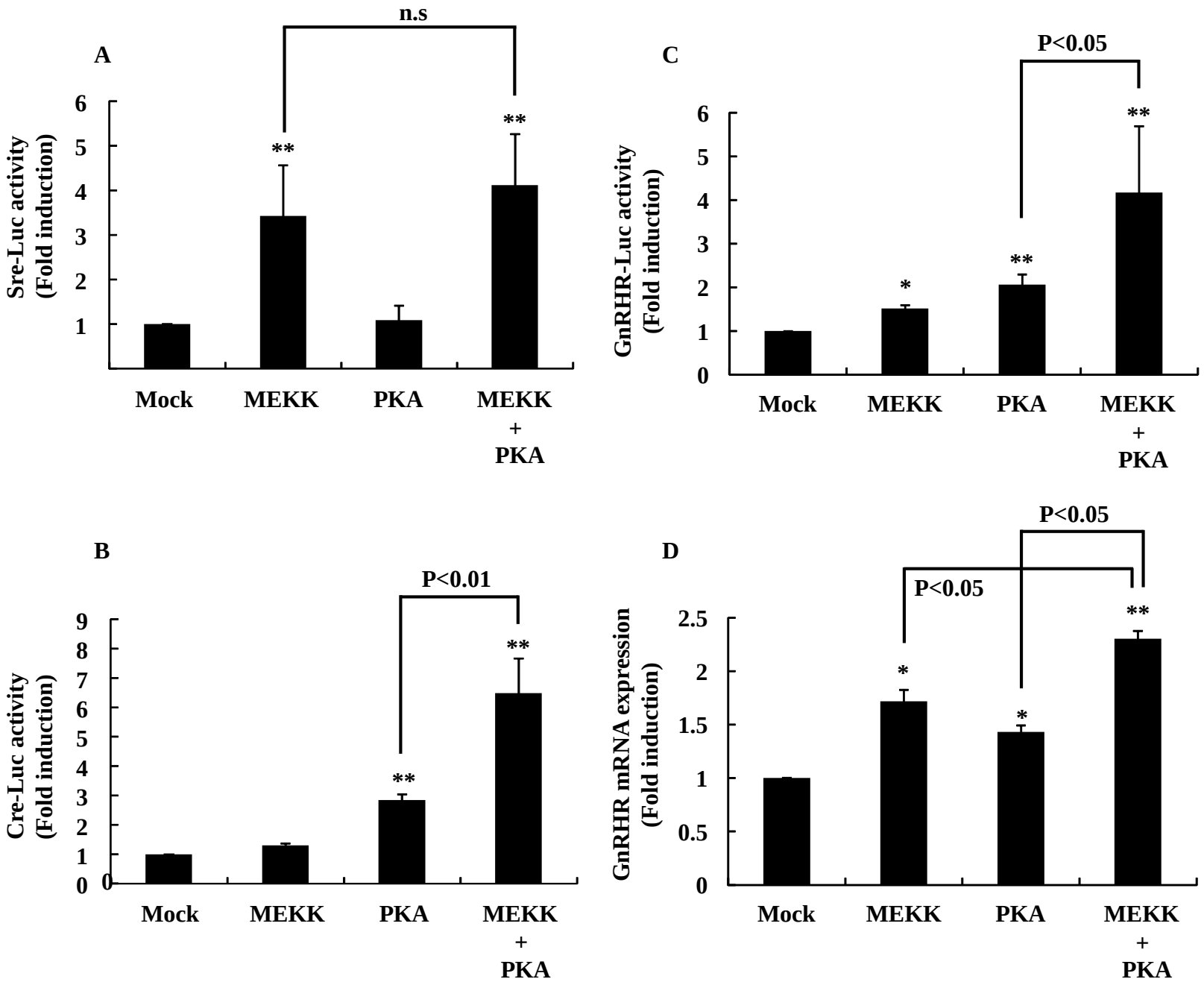
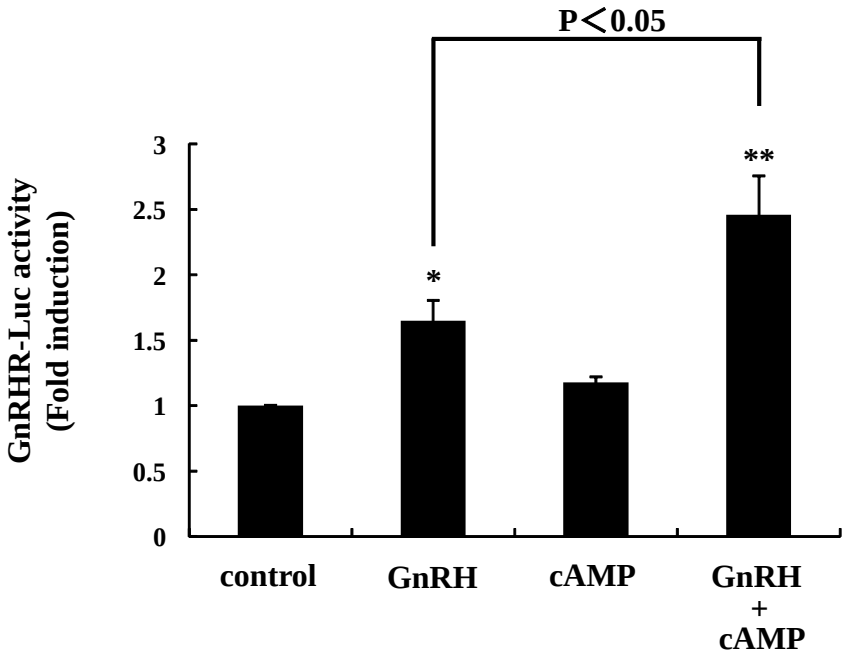

A
n.s
Sre-Luc activity
(Fold induction)
6
5
4
3
2
1
**
**
Mock
MEKK
PKA
MEKK
+
PKA
C
P<0.05
GnRHR-Luc activity
(Fold induction)
6
5
4
3
2
1
0
*
**
**
Mock
MEKK
PKA
MEKK
+
PKA
B
P<0.01
Cre-Luc activity
(Fold induction)
9
8
7
6
5
4
3
2
1
0
**
**
Mock
MEKK
PKA
MEKK
+
PKA
D
P<0.05
P<0.05
GnRHR mRNA expression
(Fold induction)
2.5
2
1.5
1
0.5
0
*
*
**
Mock
MEKK
PKA
MEKK
+
PKA

Fig 6


P＜0.05
GnRHR-Luc activity
(Fold induction)
3
2.5
2
1.5
1
0.5
0
*
**
control
GnRH
cAMP
GnRH
+
cAMP