

# 学位論文の要旨

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学位論文名 Kisspeptin Induces Expression of Gonadotropin-Releasing Hormone Receptor in GnRH-producing GT1-7 cells Overexpressing G Protein-Coupled Receptor 54

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## 論文内容の要旨

### INTRODUCTION

Reproductive functions in mammals are regulated by the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from anterior pituitary gonadotrophs, which are involved in sex steroid hormone synthesis, follicular growth and oocyte maturation. LH and FSH are mainly under the control of the hypothalamic peptide, gonadotropin-releasing hormone (GnRH). GnRH is released from hypothalamus in a pulsatile manner, and GnRH pulse frequency determines the specificity of gonadotropin synthesis and secretion. The more rapid frequencies of GnRH pulses increased the secretion of LH, whereas slower frequencies of GnRH pulses are decreased LH secretion but increased FSH secretion.

Kisspeptin, previously known 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 KiSS-1R). 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. In addition, it was found that KiSS-1- and GPR54-knockout mice exhibit infertility. Thus, kisspeptin is currently recognized as a potent stimulator of GnRH release. Furthermore, it has been reported that GnRH neurons possess their own GnRH receptor (GnRHR) and respond to GnRH. However, little is known about the direct effect of kisspeptin and GnRH on single population of GnRH neurons still largely unknown. In this study, we have investigated the action of kisspeptin using mouse GnRH-producing cell models, GT1-7. The effects of GnRH on GT1-7 cells were also examined.

### MATERIALS AND METHODS

#### *Cell culture*

GT1-7 cells, a mouse GnRH-producing cell lines or mouse pituitary LβT2 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<sub>2</sub> 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 periods.

#### ***Reporter plasmid construct and luciferase assay***

Construction of the human GPR54 expression vector and human GnRHR expression vector were transiently transfected to the cells by electroporation. An empty vector (pCI-neo) served as a mock control. When GnRHR promoter activity or the activities of the promoter containing Sre and Cre were measured, the cells were transfected together with either GnRHR-Luc, pSre-Luc or pCre-Luc vectors. Luciferase activities were measured using luminometer and determined by the ratio of firefly to the Renilla luciferase activities.

#### ***Western blotting***

The cell extracts were subjected to SDS-PAGE in 10% acrylamide gel and the protein was transferred onto polyvinylidene difluoride membranes. The membranes were incubated with phosphorylated-extracellular signal-regulated kinases (ERK) antibody. For total ERK determination, after strip washing, membranes were re-probed with anti-ERK antibody.

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

Total RNA from untreated or treated GT1-7 cells was extracted using the extraction method Trizol-S. To obtain cDNA, 1.0 µg of total RNA was reverse transcribed using an oligo-dT primer, and was prepared using a First Strand cDNA Synthesis Kit in reverse transcription (RT) buffer. Messenger RNA (mRNA) was reverse transcribed into single stranded cDNA. Quantification of GnRHR mRNA was obtained through real-time quantitative PCR using specific primer for GnRHR.

#### ***Measurement of cAMP accumulation***

Cells overexpressing GPR54 or GnRHR were plated in 96-well plates at a density of 10<sup>5</sup> 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.

#### ***Statistical analysis***

Values were expressed as means±SEM. Statistical analysis was performed using Student t-test or one-way repeated ANOVA followed by Dunnett test or Newman-Keuls test for multiple comparisons. P<0.05 was considered statistically significant.

### **RESULTS AND DISCUSSION**

To clarify whether kisspeptin and GnRH has a direct effect on GT1-7 cells, we examined the Sre- and Cre-luciferase promoters activity 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 GT1-7 cells, both kisspeptin and GnRH failed to stimulate either Sre or Cre promoter activity. Because we suspected that the expression of GPR54 and GnRHR in GT1-7 cells might have been reduced by changes in cell character due to multiple passages, cells were transfected with GPR54 or GnRHR expression vectors to determine the effect of kisspeptin and GnRH. After overexpression of GPR54 and GnRHR, both kisspeptin and GnRH increased Sre promoter activity. This observation suggests that kisspeptin and GnRH have an ability to stimulate intracellular signaling pathway when their receptors were abundantly expressed. Although Cre promoter activity was significantly activated by kisspeptin, it was not significantly increased by GnRH. That is, both kisspeptin and GnRH increased Sre promoter activity, however, the Cre promoter was increased by kisspeptin, but not by GnRH. These observations implied that GPR54, but not GnRHR has the ability to stimulate both ERK and cAMP/PKA pathways. Indeed, kisspeptin, but not GnRH increased the accumulation of intracellular cAMP in these cells. 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. On the other hand, the effect of GnRH on GnRHR promoters was limited and not significant, even when GnRHR was overexpressed. To exclude the possibility that exogenous induction of GPR54 artificially increases GnRHR expression, we overexpressed GPR54 in pituitary gonadotrophic L $\beta$ T2 cells. L $\beta$ T2 cells with overexpressed GPR54 significantly increased Cre promoter activity via kisspeptin stimulation, but no GnRHR transcriptional activity was induced by kisspeptin. Thus, we speculated that both the cAMP/PKA and ERK pathways are necessary for inducing GnRHR expression in GT1-7 cells. To further confirm this, pFC-MEKK and pFC-PKA vectors, which induce constitutively active MEKK (which ultimately stimulates ERK) and PKA were applied. Overexpression of MEKK and PKA increased GnRHR transcriptional activity as well as mRNA expression, respectively. Furthermore, combined expression of MEKK and PKA further increased GnRHR promoters. Notably, combined expression of MEKK and PKA further increased Cre promoter activity. On the other hand, PKA expression did not modify the effect of MEKK on Sre promoters. 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. 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.

### **CONCLUSION**

The present findings suggest that kisspeptin is a potent stimulator of GnRHR expression in GnRH-producing neurons. Also we speculate that both ERK and PKA activation induced by kisspeptin are necessary to stimulate GnRHR expression.