学 位 論 文 の 要 旨

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学 位 論 文 名　Action of Neurotensin, Corticotropin-Releasing Hormone, and RFamide-Related Peptide-3 in E2-induced Negative Feedback Control: Studies Using a Mouse Arcuate Nucleus Hypothalamic Cell Model

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論 文 内 容 の 要 旨

INTRODUCTION

Neurons in the hypothalamus, especially those that secrete gonadotropin-releasing hormone (GnRH), serve as the final pathway by which the brain regulates gonadotropin secretion. After the discovery of inactivating mutations in the kisspeptin receptor (Kiss1R) in families of patients with hypogonadotropic hypogonadism, it is generally agreed that hypothalamic Kiss-1 neurons, which project to GnRH neurons, control GnRH release through Kiss1R expressed in the GnRH neurons. Therefore, Kiss-1 neurons in the hypothalamus have been defined as being positioned at the highest level in the control of the hypothalamic-pituitary gonadal (HPG) axis. These neurons have been shown to be located in two different regions of the hypothalamus: the arcuate nucleus (ARC) and the anteroventral periventricular (AVPV) region in mammals. Although studies have demonstrated a bimodal effect of 17β-estradiol (E2) on the hypothalamus, having both positive and negative feedback effects on GnRH neurons that do not express estrogen receptor α (ERα), it is now postulated that Kiss-1 neurons within the ARC are the center for negative feedback by E2, whereas those in the AVPV are defined as the center of positive feedback. These conclusions are based on the observations that Kiss-1 expression in the ARC is inhibited by E2, whereas AVPV Kiss-1 expression is stimulated by E2.

Recently, a hypothalamic cell model, mHypoA-55, was established from micro dissected ARC primary cultures of female-adult-derived murine hypothalamus, which exhibits endogenous Kiss-1 expression as well as ERα and ERβ. mHypoA-55 cells also express neurokinin B (NKB), and dynorphin (Dyn), suggesting that they represent ARC kisspeptin neurons. On the other hand, mHypoA-50 cells, which were established from micro dissected AVPV primary cultures, are also known as a Kiss-1–expressing cell model that possesses ERα and ERβ. Notably, these cell

models exhibit different responses to E2 in the regulation of Kiss-1 gene expression.

In this study, we aimed to examine possible involvement of neurotensin (NT) and corticotropin-releasing hormone (CRH) in E2-induced negative feedback mechanisms using ARC hypothalamic mHypoA-55 cells.

MATERIALS AND METHODS

Cell culture

mHypoA-55 and mHypoA-50 cells were purchased from CEDARLANE LLC. Cells 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% CO2 in air. After 24 h, cells were used for each experiment. When cells were stimulated by E2, cells were cultured with E2 in phenol red–free DMEM supplemented with 1% charcoal-stripped FBS. When stimulated with the indicated neuropeptides, cells were incubated without (control) or with the test reagents in high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin for the indicated time periods.

Western blotting

The cell extracts were subjected to SDS-PAGE in 10% acrylamide gel and the protein was transferred onto polyvinylidene difluoride membranes. Membranes were incubated with anti-kisspeptin antibody, anti-NT antibody, or anti-CRH antibody.

Fetal rat brain tissue was used as positive control, and this experimental protocol was approved by the animal care and use committee of the Experimental Animal Center for Integrated Research at Shimane University

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

Total RNA from untreated or treated mHypoA50 or mHypoA55 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 *Kiss-1, NT,* and *CRH* mRNA was obtained through real-time quantitative PCR using specific primer for mouse.

Statistical analysis

Data are expressed as means±SEM. Statistical analysis was performed using Student t-test or one-way repeated (ANOVA) with the Bonferroni post hoc test, as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

First, we confirmed expression of Kiss-1 gene and kisspeptin in mHypoA-55 and mHypoA-50 cells by RT-PCR and western blotting analysis. Although both cell models express kisspeptin, these cells are distinct. mHypoA-55 cells express both Tac2 (which encodes NKB) and Dyn genes, but mHypoA-50 cells do not express these genes. To investigate responses to E2, both cell models were treated with 100nM E2, Kiss-1 mRNA expression in mHypoA-55 ARC

cells was not increased by E2; rather, it was significantly reduced at 4 h by E2 and returned to the control level at 24 h. Contrary, in mHypoA-50 AVPV cells, Kiss-1 mRNA expression increased by E2 to 3.03 ± 0.54- fold at 24 h.

Next, we confirmed expression of NT and CRH by western blotting and RT-PCR analysis in both cell models. By NT stimulation, Kiss-1 mRNA expression was significantly decreased to almost half that of untreated cells in mHypoA-55. Similarly, when mHypoA-55 cells were stimulated by CRH, Kiss-1 mRNA expression was significantly reduced to 0.62 ± 0.17-fold at 100 nM compared to untreated control. This result implies that NT and CRH could repress Kiss-1 gene expression in hypothalamus Kiss-1 neurons located in ARC region.

Both NT and CRH expression in mHypoA-55 cells were influenced in the presence of E2. By 24 h E2 stimulation, NT expression was significantly increased to 1.71 ± 0.19- fold, and CRH expression were increased to 5,89 ± 1.87-fold in mHypoA-55. These observations suggest that NT and CRH expressions in the hypothalamus are upregulated by E2.

RFamide-related peptide-3 (RFRP-3) is a mammalian homolog of gonadotropin-inhibitory hormone which was first identified as a neuropeptide that inhibits gonadotropin release in birds. We found that the RFRP-3 gene is expressed in mHypoA-55 cells. Similar to the phenomenon observed in birds, 10 μM melatonin stimulation increased RFRP-3 mRNA expression to 1.93 ± 0.39-fold in mHypoA-55 cells. In addition, RFRP-3 gene expression in mHypoA-55 cells was slightly but significantly increased by E2 to 1.55 ± 0.19-fold. Furthermore, NT mRNA expression was significantly increased by RFRP-3 to 1.94 ± 0.24-fold at 100 nM and CRH mRNA expression significantly increased to 2.39 ± 0.72- fold at 10 nM of RFRP-3. These observations implied that both NT and CRH were regulated under the influence of RFRP-3.
Furthermore, we found that melatonin, which had the ability to stimulate RFRP-3 expression, increased the NT and CRH gene expression by 1.55 ± 0.21-fold and 2.96 ± 0.54- fold, respectively.

Lastly, we examined the effect of NT and CRH on mHypoA-50 AVPV cell models. When mHypoA-50 cells were stimulated with E2 for 24 h, NT expression was significantly increased by 1.71 ± 0.19-fold. Similarly, CRH mRNA was increased by E2 to 3.74 ± 0.93-fold in mHypoA-50 cells. However, in contrast with NT inhibition of Kiss-1 gene expression in mHypoA-55 ARC cells, 10 nM NT significantly increased Kiss-1 mRNA expression to 3.63 ± 1.45-fold in mHypoA-50 AVPV cells. Similarly, Kiss-1 mRNA expression was significantly increased by CRH stimulation in mHypoA-50 cells to 1.75 ± 0.29- fold. These NT and CRH effects on Kiss-1 gene expression in mHypoA-50 AVPV cells were completely opposite of those observed in mHypoA-55 ARC cells.

CONCLUSION

Our observations using the hypothalamic ARC cell model mHypoA-55 suggest that NT and CRH have inhibitory effects on Kiss-1 gene expression under the influence of E2 in association with RFRP-3 expression. Thus, these neuropeptides might be involved in E2-induced negative feedback mechanisms.