論文の内容の要旨

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Role of arcuate dynorphin-kappa opioid receptor signaling in regulating pulsatile luteinizing hormone secretion in female rats
（メスラットのパルス状 LH 分泌における弓状核ダイノルフィンーカッパオピオイド受容体シグナリングの役割）

In mammal species, reproduction is regulated by the hypothalamic–pituitary–gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) is one of the most important neuropeptides secreted from the hypothalamus, which acts to trigger the secretion of luteinizing hormone (LH) from the pituitary. Both GnRH and LH are secreted in two patterns: the pulse and the surge. It is now well known that GnRH/LH surge is in charge of ovulation while the pulses, especially the frequency of the pulses, are of great importance for estrous cycles, follicular development and sex steroid hormone secretion.

In 2003, kisspeptin, together with its receptor GPR54, were found to have a stimulatory effect on the reproduction system. Evidence including the regulatory role of kisspeptin on LH secretion, expression of steroid hormone receptors in kisspeptin neurons and expression of GPR54 in GnRH neurons have been found one by another in the past decade, proving that kisspeptin is the upstream regulator of GnRH secretion. Among neural nuclei that express kisspeptin, kisspeptin neurons in the arcuate nucleus (ARC) co-express neurokinin B (NKB) and dynorphin A (Dyn), which led to the acronym KNDy neurons. This cluster of kisspeptin neurons is also considered to be the possible GnRH pulse generator. This idea first came from a hypothalamic deafferentation experiment, which proved the GnRH pulse generator to be located in the mediobasal hypothalamus (MBH). This hypothesis is further confirmed by experiments that recorded periodic multiple-unit activity (MUA) volleys from the ARC.

NKB and Dyn are thought to be two important regulators in pulse generation. NK3R, the receptor of NKB, has been demonstrated to be expressed in KNDy neurons. Therefore, NKB/NK3R system was suggested to play a role in an auto- or paracrine feedback loop. This NKB/NK3R signaling pathway is thought to stimulate neuronal activity and synchronize firing of KNDy neurons. On the other hand, Dyn

and kappa-opioid receptor (KOR, a receptor for Dyn) has been proved by several experiments to suppress pulsatile LH secretion. By possessing these two opposite signaling pathways that control KNDy neurons in a see-saw mechanism, KNDy neurons seem to acquire the ability to become a pulse generator.

However, Unlike NK3R, the distribution of KOR has not been verified with solid proof. Little is known now about how dynorphin works in the regulation of pulse generation, nor the character of the cells that express Oprk1, the gene that encodes KOR, in the ARC. The present dissertation aims to discuss the following three questions: 1) identification of Oprk1-expressing cells in the ARC; 2) the role of Oprk1-expressing cells in regulating GnRH pulse generation and 3) the signaling pathway of Dyn-KOR to KNDy neurons.

In chapter 2, identification of Oprk1-expressing cells was discussed. Distribution of KOR in the brain was first described in 1994 using a combination of radioactive in situ hybridization (ISH) and receptor autoradiographic techniques. Little progress was made since then, because GPCRs, such as KOR, are usually expressed at a low level and share conserved structures, which makes them difficult to be detected in situ. Although some recent studies reported that Oprk1 was expressed in KNDy neurons in mice, this conclusion only depended on some low-resolution double staining pictures with Oprk1 detected by radioactive ISH.

Therefore, for the study of the role of Oprk1-expressing cells in regulating GnRH/LH pulse generation, it is important to clarify its relationship with kisspeptin neurons. By optimizing the protocol, the current study successfully detected Oprk1 mRNA by fluorescent ISH and double stained Oprk1 and kisspeptin in the ARC. Results showed the staining of Oprk1 and kisspeptin throughout the ARC, but no Oprk1 mRNA was detected in kisspeptin neurons. On the other hand, axons from kisspeptin neurons projecting to the Oprk1-expressing cells were detected in the ARC.

Results in chapter 2 demonstrated that Oprk1-expressing cells in the ARC are different from KNDy neurons. Although different from the previous studies on the identification of Oprk1-expressing cells, staining method used in the current study succeeded to describe the contour of Oprk1-expressing cells and was able to separate signals from cells in different layers in the tissue. As an evidence proving that the Oprk1-expressing cells detected in this study were involved in the regulation of LH pulse generation, axons from kisspeptin neurons were detected to project to Oprk1-expressing cells. This projection is speculated to be Dyn to form the Dyn-KOR signaling and act as a regulator of LH pulse generation.

Chapter 3 focused on the role of Oprk1-expressing cells in regulating pulsatile LH secretion. As results in chapter 2 demonstrated that Oprk1-expressing cells are different from KNDy neurons in the ARC, a

new neural circuit of Dyn-KOR signaling mediating kisspeptin secretion was brought forward. Considering that little is known about the function of this Oprk1-expressing cell, it is of great importance to value its role in regulating GnRH pulse generation. Besides, even though there were many studies investigating the effect of Dyn on LH secretion using KOR agonists and antagonists, we still need more knowledge about the neural pathway within and downstream to Oprk1-expressing cells.

In chapter 3, I took use of a cytotoxin to investigate the function of Oprk1-expressing cells in regulating the pulse generation. This cytotoxin, Dyn-SAP, was made toxic only to Oprk1-expressing cells while non-harmful to other cells. By administrating this drug into the ARC, effects of ablation of Oprk1-expressing cells on pulsatile LH secretion were examined.

Both in vitro and in vivo experiments demonstrated that Dyn-SAP is an effective and selective cytotoxin to Oprk1-expressing cells. Three weeks after the intra-ARC drug administration, about half of Oprk1-expressing cells were ablated. As a result, LH pulse frequency increased significantly from 6.50 ± 0.61 times/3h (control group) to 8.43 ± 0.30 times/3h (Dyn-SAP administrated group), and baseline of plasma LH concentration also increased significantly (SAP 0.87 ± 0.09 ng/ml vs. Dyn-SAP 1.12 ± 0.06 ng/ml, $p < 0.05$). On the other hand, kisspeptin neurons in the ARC did not decrease.

The study in chapter 3 demonstrated that Oprk1-expressing cells in the ARC is involved in inhibiting LH pulse generation because lesion of Oprk1-expressing cells caused an increase of LH pulse frequency and baseline concentration. Therefore, it is reasonable to conclude that the output from Oprk1-expressing cells to KNDy neurons might be a “negative signal” to suppress the activity of KNDy neurons. Besides, the number of kisspeptin neurons showed no significant difference between Dyn-SAP- and SAP-groups, suggesting that Dyn-SAP does not harm neurons except for those express Oprk1. This result also proved that Oprk1 is not expressed in kisspeptin neurons from other perspectives.

In chapter 4, I tried to investigate the response of Oprk1-expressing cells to Dyn. This idea came from the conflict between the conclusion in chapter 3 and previous studies injecting Dyn or KOR agonist. In chapter 3, it was concluded that signals secreted from Oprk1-expressing cells to KNDy neurons suppress kisspeptin secretion. Considering that Dyn or KOR agonist have been proved to suppress LH pulses, it is reasonable to speculate that Oprk1-expressing cells are activated by binding to its ligands. But this inference is contradictory to the existing theory about the G protein coupled with KOR. The G protein coupled with KOR is Gi type α subunit, which is known for their suppressive effect to decrease cell activity. Hence, we can infer that some ideas about the pathway in Dyn-KOR signaling need to be revised.

In experiments in this chapter, a specific KOR agonist, nalfurafine, was injected into the third ventricle of adult female Wistar-Imamichi rats. Brains were sampled one hour after the injection and c-Fos was stained together with Oprk1 as an indicator of the activity of Oprk1-expressing cells. Results showed that the number of double-stained cells in the ARC of agonist-administrated group was significantly higher than that of vehicle-administrated control group.

This study demonstrated that Oprk1-expressing cells in the ARC were activated by nalfurafine. c-Fos is commonly accepted as an indicator of neural activity, but the intracellular signal pathway downstream from Gαi is still under debate. Although it was generally accepted that KOR inactivate cells by activating a G protein-coupled inwardly-rectifying potassium channel (GIRK), which hyperpolarize cells, more and more studies have demonstrated that Gαi can also inactivate GIRK. This suppression of GIRK may results in the activation of Oprk1-expressing cells and the increase of c-fos-immunopositive-Oprk1-expressing cells in this study. Another possible cascade is the MAPK pathways. Two classes of MAPK, the ERK and p38 MAPK pathway, have been proved to be activated by KOR. These two classes of MAPK were demonstrated to increase c-fos gene expression and stabilize c-Fos protein by phosphorylation.

As the conclusion of the present dissertation, the series of experiments demonstrated that Oprk1-expressing cells in the ARC is different from kisspeptin neurons. These cells receive projection from kisspeptin neurons and suppress KNDy neuron activity by secreting “negative signals” in return. The present results also brought new ideas to the issue considering the signal pathway inside Dyn-KOR signaling that Oprk1-expressing cells would be activated by Dyn. But considering the multiple roles of Gαi and GIRK pathways, especially in different cell types, more studies are necessary to clarify the exact signaling in Oprk1-expressing cells.