博士論文

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―カッパオピオイド受容体シグナリングの役割）

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Abstract

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.

Table of Contents

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The Author

CHAPTER 1

General introduction

Hypothalamic–pituitary–gonadal axis regulates reproductive functions

In mammal species, reproduction is regulated by the hypothalamic–pituitary–gonadal (HPG) axis. Even though the first realization of the relationship between reproduction and brain came in the middle of 19th century, it is not easy to figure out this axis and, much is still remained in mystery even in nowadays. Luteinizing hormone (LH), secreted by the pituitary, is one of the most important reproductive hormones [1]. The hormone has two patterns of secretion, the pulse and the surge. Pulsatile LH is a series of periodic abrupt increase in LH concentration followed by an exponential decrease [2], which is necessary for the tonic support of reproductive functions [3–5] including initiation of estrous cycles, follicular development and sex steroid secretion, while the surge acts as a signal for ovulation [6]. Later on, gonadotropin-releasing hormone (GnRH), secreted from the hypothalamus, was proved to be the upstream of LH [7–9]. As the exclusive neuropeptide from the hypothalamus to trigger pulsatile LH secretion [10,11], GnRH was also considered to be secreted in a pulsatile pattern as LH. This was proved by portal cannulation in sheep and very frequent portal blood collections showing GnRH pulses, which correspond to LH pulses [12,13]. Additional researches demonstrated effects of artificial pulsatile GnRH infusion on restoration of LH pulses [14] and menstrual cycles [15].

Kisspeptin neurons are headquarter of HPG axis

Even with the discovery of GnRH, researchers have kept tracing the headquarters of reproductive regulator. Previous studies have proved that both the secretion of LH [16,17] and GnRH [7–9] are controlled by the feedback of steroid hormones.

Considering the fact that no estrogen receptor α (ERα), progesterone receptor (PR) or androgen receptor is expressed in GnRH neurons [18–20], there seemed to be another "missing link" upstream to GnRH neurons that mediates the steroid hormone feedback action.

In 2003, a neuropeptide previously called metastin was found to have a tonic effect on the reproduction system [21,22]. This neuropeptide, now known as kisspeptin, was suggested to be the master regulator of GnRH secretion (Fig. 1-1). Effects of kisspeptin in regulating the secretion of LH and GnRH have been proved in a wide variety of species [23,24]. Expression of steroid hormone receptors in kisspeptin neurons [25,26] instead of GnRH neurons suggested a function of kisspeptin neurons mediating the feedback control of GnRH /LH secretion. Because GPR54, a receptor for kisspeptin [21,22], belongs to G protein-coupled receptor super-family [27], it has been difficult to localize it with immunostaining method. Until recently, only anatomical studies have shown that kisspeptin fibers connect and closely adjacent to GnRH neurons in the preoptic area (POA) and GnRH axons in the median eminence (ME) [28–30]. Therefore, kisspeptin could affect GnRH secretion directly. Studies using a transgenic *Gpr54* LacZ knock-in mouse model provided the first solid *in vivo* proof showing that *Gpr54* co-expresses in GnRH neurons [31]. Later in 2015, Higo et al achieved the first fluorescent in situ hybridization (ISH) of *Gpr54* and double stained *Gpr54* and GnRH in the same neuron [32], demonstrating that kisspeptin regulates HPG axis directly through GnRH neurons (Fig. 1-1).

KNDy neurons are conceivable pulse generator

In 1970, Knobil’ s work on the rhesus monkey revealed, for the first time, the

pulsatile secretion pattern of luteinizing hormone (LH) [2]. This result led to a shift in the perception of hormone actions that the gonadal activity is controlled by the frequency of LH pulses [4,33,34]. Therefore, manipulation of the activity of the GnRH/LH pulse generator is of both theoretical and therapeutic importance. Early in 1974, a hypothalamic deafferentation experiment had proved that the GnRH pulse generator located in the mediobasal hypothalamus (MBH) [35], which includes the arcuate nucleus (ARC). This experiment also brought the idea that GnRH pulse generator may not include GnRH neurons themselves because most of the GnRH neurons are located outside of MBH in most animal species.

As an upstream regulator of GnRH secretion, kisspeptin naturally got the attention to be a potential pulse generator. *Kiss1*, the gene coding kisspeptin, is highly expressed in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) [36,37]. Following the deafferentation experiment, Knobil's laboratory first recorded the multiple-unit activity (MUA) from MBH, which was corresponding to changes in LH pulses [38]. This record of neural activity, together with other studies in other animals [39–41], demonstrated that the pulsatile secretion of GnRH is governed by neurons in the MBH. The MUA experiment in goats carried out by Wabayashi et al [42] furtherly linked ARC- kisspeptin neurons to the pulse generator, as their results showed that the MUA in the ARC is regulated by steroid hormone.

The majority of ARC kisspeptin neurons co-express two other neuropeptides implicated in the control of reproduction [43,44], neurokinin B (NKB) and dynorphin A (Dyn). This colocalization is highly conserved across species [42,45–47], leading to the acronym KNDy (Kisspeptin/NKB/Dynorphin) neurons [48] (Fig. 1-1). Anatomical evidence proposed KNDy neurons to be the GnRH pulse generator.

Experiments have already showed that KNDy neurons comprise a neuronal network interconnected by axon collaterals [49,50]. The fact that KNDy neurons also contain NKB receptors (NK3R) suggests that NKB/NK3R system plays a role in an auto-feedback or paracrine-feedback loop [49] (Fig. 1-1). This NKB/NK3R signaling pathway is thought to stimulate neuronal activity and synchronize firing of all the neurons [51]. On the other hand, Dyn is considered to participate in suppressing neuronal activity, which, in other words, stops KNDy neurons firing [42].

Dynorphin is a regulator of GnRH/LH pulses and key factor in pulse generation

Dyn and kappa-opioid receptor (KOR, a receptor for Dyn) has been proved by several experiments to suppress pulsatile LH secretion. Simon et al. showed that Dyn inhibits the firing rate of ARC kisspeptin neurons via KOR *in vitro* in mouse brain slice [52]. The administration of Dyn suppressed LH pulses and MUA volleys and BNI, a KOR antagonist, stimulated MUA volleys in goats [42]. Mostari et al. showed that Dyn-KOR signaling mediates the estrogen negative feedback action on GnRH/LH pulses [53]. These studies raise the possibility that Dyn-KOR signaling negatively controls pulsatile GnRH/LH secretion.

Dyn-KOR signaling was considered to play an important role in generating GnRH pulses. Unlike NKB, Dyn is a neuropeptide acts to suppress neuronal activity. By possessing the two opposing signaling mechanisms, NKB/NK3R and dynorphin/KOR signaling, KNDy neurons seem to acquire the ability to become a pulse generator [42].

Objective

Unlike NK3R, the distribution of KOR has not been verified with solid proof [54].

According to unpublished results done by the Tsukamura group in Nagoya University, *Oprk1*, the gene that encodes KOR, is not expressed in the KNDy neuron (unpublished results). Little is known now about how dynorphin works to regulate GnRH pulse generation, nor the character of *Oprk1*-expressing cells. This study aims to answer 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.

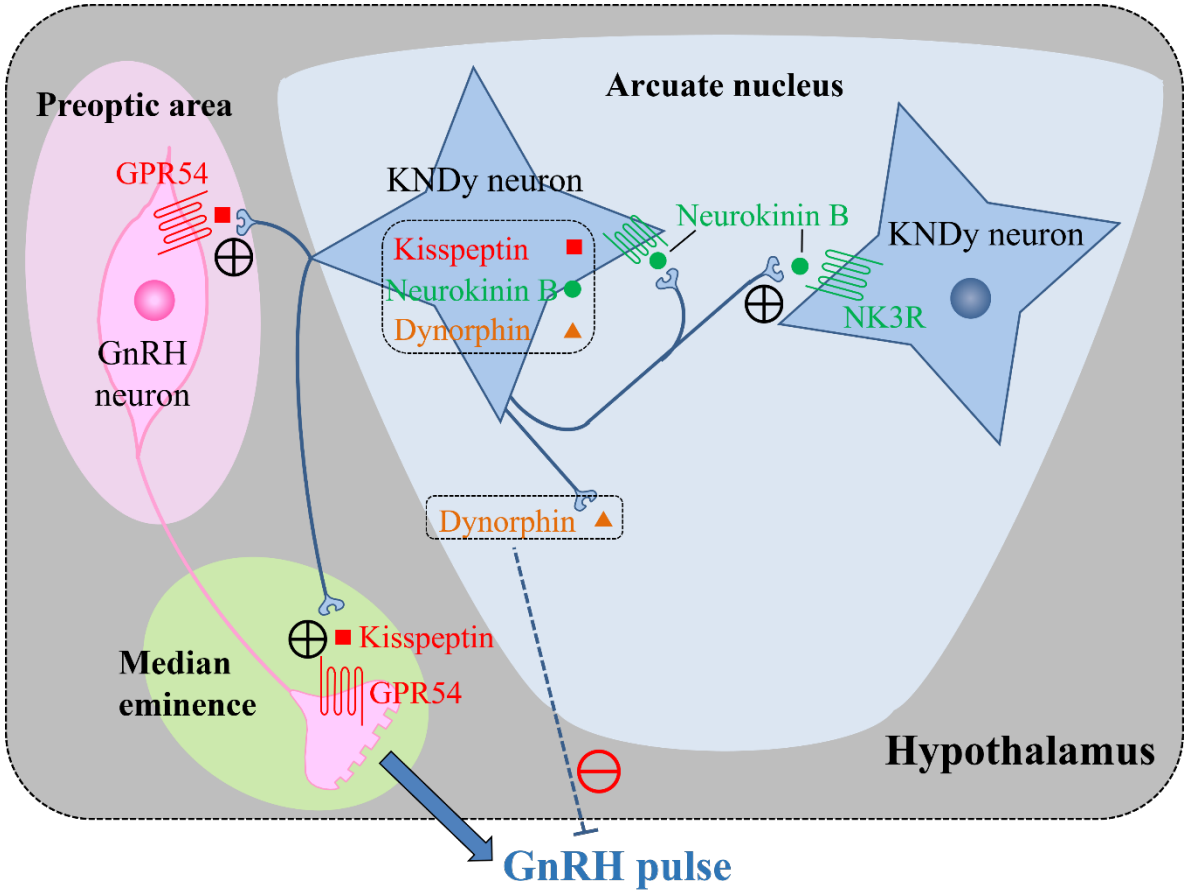


Figure 1-1. Schematic illustration of neural circuit involves KNDy neurons and GnRH neurons in the hypothalamus for the regulation of GnRH pulses. KNDy neurons in the ARC secrete kisspeptin to the GnRH cell bodies and presynaptic terminals to promote GnRH secretion. KNDy neurons form a neural circuit by their collaterals and dendrites. NKB/NK3 signaling plays the role of accelerator in autocrine or paracrine manner. Dynorphin suppress pulsatile GnRH secretion, but the neural circuit downstream to dynorphin is still unclear. Dotted line with a short bar at the end represent indirect suppressive effect. The plus mark inside a circle represent promoting effect, while minus mark inside a circle means suppress. KNDy neuron, kisspeptin/NKB/Dyn neuron.

CHAPTER 2

Characterization of *Oprk1*-expressing cells in the ARC

Introduction

KOR is a G protein-coupled receptor (GPCR) encoded by *Oprk1* gene in chromosome 5 in rats [55]. It is one of the four major opioid receptors [56], which binds dynorphin as the primary endogenous ligand. Similar to other opioid receptors, KOR is rich in the central nerve system [55], including hypothalamus, periaqueductal gray, claustrum and spinal cord [57,58]. It is responsible for mediating several physical activities like nociception, consciousness, depression and stress.

Distribution of KOR in the brain was first described in 1994 using a combination of radioactive *in situ* hybridization (ISH) and receptor autoradiographic techniques [54]. Both two methods confirmed the existence of KOR in several brain regions including the endopiriform nucleus, claustrum, olfactory tubercle, medial preoptic area, paraventricular and ventromedial hypothalamic nuclei, arcuate nucleus and so on. The research, however, provided only some low magnification photos and failed to verify the cell type that express *Oprk1*. The identity of *Oprk1*-expressing cells in the ARC is still in the dispute to date.

The absence in the identification of KOR-containing cells is due to its character as a GPCR. GPCRs are usually expressed at a low level [55], which makes it difficult to be detected by nonradioactive ISH. GPCR is a superfamily of receptors characterized by having an extracellular N-terminus, 7-transmembrane spanning domains (TMDs) and an intracellular C-terminus [59]. This superfamily can be divided into several subfamilies to make a number of characteristic motifs of the TMD that are highly conserved within the subfamilies. This similarity in structure and the problems in getting suitable antigen reagents for antibody generation [60] make it difficult to produce anti-serum with the high specificity to each GPCR.

A recent study reported that *Oprk1* is expressed in KNDy neurons in mice [61]. Nevertheless, this conclusion only depended on some low-resolution double staining pictures with *Oprk1* detected by radioactive ISH. Another problem considering the reliability of this report is that radioactive ISH cannot distinguish signals from different layers of the tissue, so that this method is in fact not suitable for judging whether two signals came from the same cell.

For the study of the role of *Oprk1*-expressing cells in regulating LH/GnRH pulse generation, it is important to clarify its relationship between kisspeptin neurons. By optimizing the protocol, the current study successfully detected *Oprk1* mRNA by fluorescent ISH and double stained *Oprk1* and kisspeptin to clarify whether *Oprk1* expresses in kisspeptin neurons in the ARC.

Materials and Methods

Animals and treatment

Young adult, female Wistar-Imamichi rats (8-week old at the time of ovariectomy, Institute for Animal Reproduction, Ibaraki, Japan,) were individually housed in a quiet, temperature- (22 ± 2 °C) and humidity-controlled room in the University of Tokyo with a 14-h light, 10-h dark cycle (lights on at 0500 h). Rats had a free access to water and food (CE-2; Clea Japan Inc., Tokyo, Japan). After two normal estrous cycles were confirmed by vaginal smear, rats (n=3) were bilaterally ovariectomized (OVX). Twelve days after OVX, rats received brain surgeries to inject colchicine into the lateral cerebroventricle following the protocol in our previous reports [62]. All the surgeries were performed under isoflurane anesthesia. All animals were injected with antibiotics (Mycillin; Meiji Seika, Tokyo, Japan) after any surgeries. All protocols were conducted under the “Guidelines for Proper Conduct of Animal Experiments” from the University of Tokyo.

Nissl staining and double staining of Oprk1 and kisspeptin

Two days after brain surgery, brain samples were collected from rats which were transcardially perfused with 50 ml of phosphate-buffered saline (PBS; 0.05 M phosphate buffer, 0.1 M NaCl, pH 7.4) followed by 50 ml of 4% paraformaldehyde in 0.05M phosphate buffer (PB, pH 7.4). Brain samples were post-fixed in the same fixative at 4°C for 16 h, cryoprotected in 30% sucrose in PB for 48 h and frozen in Tissue-Tek (Sakura Finetek, Tokyo, Japan) at -20°C for sectioning. Four series of serial coronal sections at 50-μm thickness were prepared using a cryostat (Leica CM1860, Heidelberg, Germany) and kept in cryoprotectant (0.5 M sucrose, 6.4 mM

$MgCl_2$, 0.1 M NaCl, 0.05 M PB, 50% glycerol, pH 7.4) at -30 °C before use.

For Nissl staining, coronal brain sections from each individuals were stained with thionin solution. Thionin-stained brain sections underwent microscopic inspection (DM 2500, Leica Microsystems, Wetzlar, Germany) for confirmation of the site of i.c.v injection of colchicine. Only data from subjects in which brain surgery was verified to be successful were subjected to other analyses.

Oprk1 mRNA was detected by free-floating *in situ* hybridization using the DIG-labeled antisense cRNA probe aiming position 264-1404 of NM_001318742. Immunofluorescence (IF) was also carried out with the same set of sections to stain kisspeptin protein. Brain sections were first washed by PBS, followed by 1% H_2O_2 in PBS treatment to remove endogenous peroxidase. Sections were then incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature. Pre-hybridization was done in hybridization-buffer containing 50% formamide and 4% dextran sulfate for 1 h at 60 ºC. For hybridization, sections were incubated with cRNA probes at a concentration of 1μg/ml in hybridization-buffer for 16 h at 60 °C. For the linearization, the probes had been preheated in hybridization-buffer for 3 minutes at 85 ºC and chilled on ice for 5 minutes prior to hybridization.

After hybridization, sections were rinsed in 4 × SSC containing 50% formamide. Sections were then treated with 20 μg RNase A (Roche Diagnostics, Mannheim, Germany) diluted in 1 ml TNE buffer for 30 min at 37 ºC. The RNase was incubated at 80 ºC for 30 min to inactivate DNase according to the manufacture's instruction. Sections were rinsed in 2 × SSC followed by 0.5 × SSC and then subjected to blocking with 1 ml of TBS buffer containing 1.0 % Blocking Reagent (Roche Diagnostics). After the blocking, brain sections for the staining of *Oprk1* mRNA were then

incubated with Anti-Digoxigenin-POD antibody (Roche Diagnostics, Mannheim, Germany) at a dilution of 1:500 in 1 ml blocking reagent for 1.5 h at room temperature. TSA biotin detection kit (PerkinElmer, Boston, USA) was used to bring biotin into this system.

Before coloration of *Oprk1* mRNA, IF was first done. Sections treated with TSA biotin detection kit were blocked in 10% normal goat serum for 1 h and then incubated with rabbit anti-mouse kisspeptin antibody (C2, produced and provided by Dr. Okamura in The National Institute of Agrobiological Sciences). A goat anti-rabbit IgG conjugated to fluorescence (Alexa594, Thermo Fisher Scientific, USA) 2nd antibody was used to coloration of kisspeptin. Sections were incubated with Streptavidin Alexa Fluor 488 conjugate (Invitrogen, Eugene, USA) for 1.5 h at a dilution of 1:200 for coloration of *Oprk1* mRNA and finally mounted on gelatin-coated slides and embedded with Prolong Gold (Life Technologies Co., Tokyo, Japan) for analysis by LSM 700 laser scanning microscope (Carl Zeiss, Inc. Thornwood, USA).

Results

Verification of the location of i.c.v colchicine injection

Traces of the i.c.v injection of colchicine were verified with Nissl-stained brain sections. All animals appear to have a similar site of injection, which was located 0.8 mm posterior to bregma as showed in the representative picture in Fig. 2-1. No destruction of brain tissues but the cannula trace pierced from the top of brain into lateral ventricle was observed.

Kisspeptin neurons in the ARC do not co-express Oprk1

Brain sections containing ARC (from about 1.8 mm posterior to bregma to 4.3 mm posterior to bregma) from all individuals (n=3) were checked for the signal of *Oprk1* and kisspeptin. As representative photos shown in Fig. 2-2, the staining of *Oprk1* or kisspeptin can be seen throughout the ARC. Among all the sections from the three individuals, no signal of *Oprk1* mRNA was detected in kisspeptin neurons.

Fig. 2-3 shows an example of the overlapping of *Oprk1*-expressing cells and kisspeptin neurons. The staining of *Oprk1* and kisspeptin locate in similar position. But both *Oprk1* and kisspeptin signals were found to located in some areas exclusively.

Kisspeptin neurons project to Oprk1-expressing cells in the ARC

In sections from all individuals, some kisspeptin-immunopositive axons were found located adjacent to *Oprk1*-expressing cells in the ARC (Fig. 2-4). Some of these axons can be confirmed to be projected from kisspeptin neurons in the ARC. Therefore, it was considered that kisspeptin neurons in the ARC, at least part of them, project to ARC-*Oprk1*-expressing cells.

Discussion

In the present study, *Oprk1*-expressing cells in the ARC were demonstrated to be different from KNDy neurons. The OVX animal model used in the experiments has been proved to have a relative high expression level of both *Oprk1* and *Kiss1* [47]. Injection of colchicine is a general technique used to stain cell bodies containing secreting neuropeptides like kisspeptin, as demonstrated by previous studie [62]. In order to decrease the influence on the following staining, no dye was injected together with colchicine. But the location of injection trace (Fig. 2-1) proved that injections were carried out in correct location. Three individuals with normal estrous cycle before OVX were used, and sections from them was checked thoroughly for the merge of *Oprk1* mRNA signal and kisspeptin immunostaining. Therefore, it is reasonable to conclude that there is no *Oprk1* expression in KNDy neurons.

Previous studies using radioactive ISH [61] for the staining of *Oprk1* reported a co-expression of *Oprk1* and kisspeptin. This study, however, only provided some low-resolution pictures. The silver grain used in that study can only represent cell bodies by a cluster of white dots, unable to show the clear shape of a cell. Another important problem for the method used in that paper was that radioactive ISH cannot distinguish signals from different layers of the tissue. This means signals from two vertically overlapping cells may be considered coming from the same one. In fact, overlapping kisspeptin neuron and *Oprk1*-expressing cell as shown in Fig. 2-3 were detected in the present study. Staining method used in this study was able to describe the contour of cells. Therefore, it is able to tell that signals pointed by arrow and arrowhead in Fig.2-3 represent two different cells.

Some other studies also mentioned the concept that *Oprk1* is expressed in KNDy

neurons [63,64]. But this theory mostly depends on the results of the previous study using radioactive ISH, or speculation based on the function of Dyn-KOR signaling as a pulse generator. Possessing the two opposing signaling mechanisms, NKB/NK3R and Dyn/KOR was thought to be the condition for KNDy neurons to become a pulse generator. Therefore, similar to the autocrine or paracrine loop of NKB/NK3R signaling in the ARC, KOR was believed to exist in KNDy neurons. Therefore, Dyn, secreted together with kisspeptin during the firing of pulses, can suppress the activity of KNDy neurons directly and thus form the exponential decrease of LH. However, according to the results in the present experiments, Dyn is probably not acting directly on KNDy neurons. The current result provides the possibility that some unidentified "*Oprk1*-expressing cells" in the ARC may mediate the suppressing signal of Dyn to cease the firing of KNDy neurons.

In the present study, axons from kisspeptin neurons were detected projecting to *Oprk1*-expressing cells. This could be an evidence indicating that the *Oprk1*-expressing cells were involved in the regulation of GnRH pulse generation. The red line in Fig. 2-4 pointed by arrows represent the axon from a kisspeptin neurons, which are adjacent to *Oprk1*-expressing cells (marked by arrowheads).

The present study demonstrated that *Oprk1*-expressing cells in the ARC are different from kisspeptin neurons. Different from previous hypothesis, this new identified *Oprk1*-expressing cell, instead of KNDy neurons in the ARC, receives the projection from KNDy neurons. This projection is speculated to be Dyn to form the Dyn-KOR signaling and act as a regulator of LH pulse generation.

In this paper, authors achieved non-radioactive *in situ* hybridization of *Oprk1* for the first time. This skill will be used in future research to provide more solid proof

considering the cell type that express *Oprk1*.

Figures

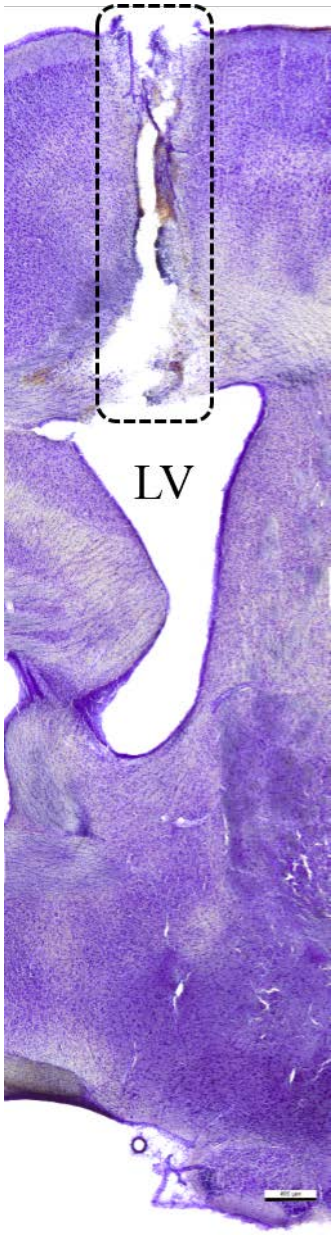


Figure 2-1. Verification of the i.c.v injection location (Scale bar: 400 μm). Black dotted line shows the trace of i.c.v cannula. LV, lateral ventricle.

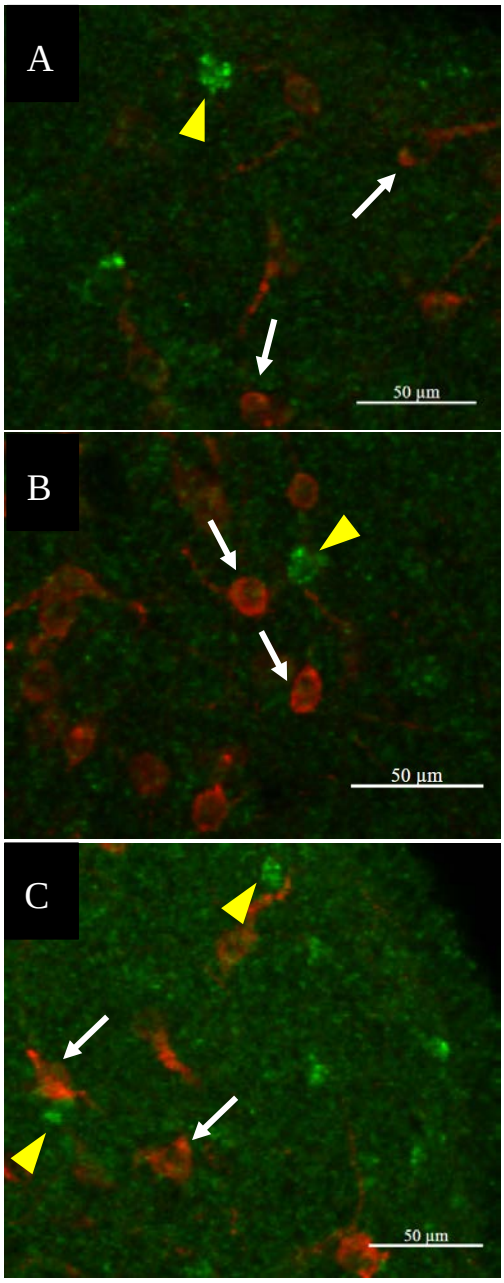


Figure 2-2. Representative photos for immunofluorescent of kisspeptin neurons (red, marked by white arrows) and *in situ* hybridization of *Oprk1*-expressing cells (green, marked by yellow arrowheads) in the ARC. Figure A, B and C are photos from anterior, middle and posterior ARC, respectively. Scale bars: 50 µm.

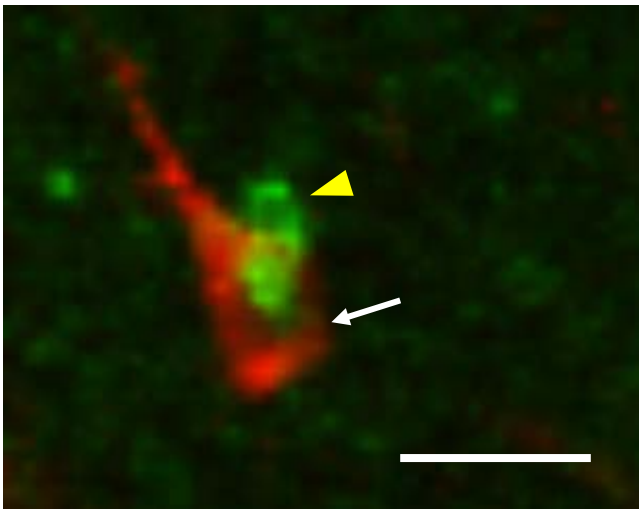


Figure 2-3. Representative photo for the overlapping of kisspeptin neurons and *Oprk1*-expressing cells in the ARC. The green stained cell marked by yellow arrowhead is an *Oprk1*-expressing cell. White arrow marks a kisspeptin neuron with red immunofluorescence. About half of the *Oprk1*-expressing cell overlaps with the kisspeptin neuron while the other half is not. Scale bars: 20 μm.

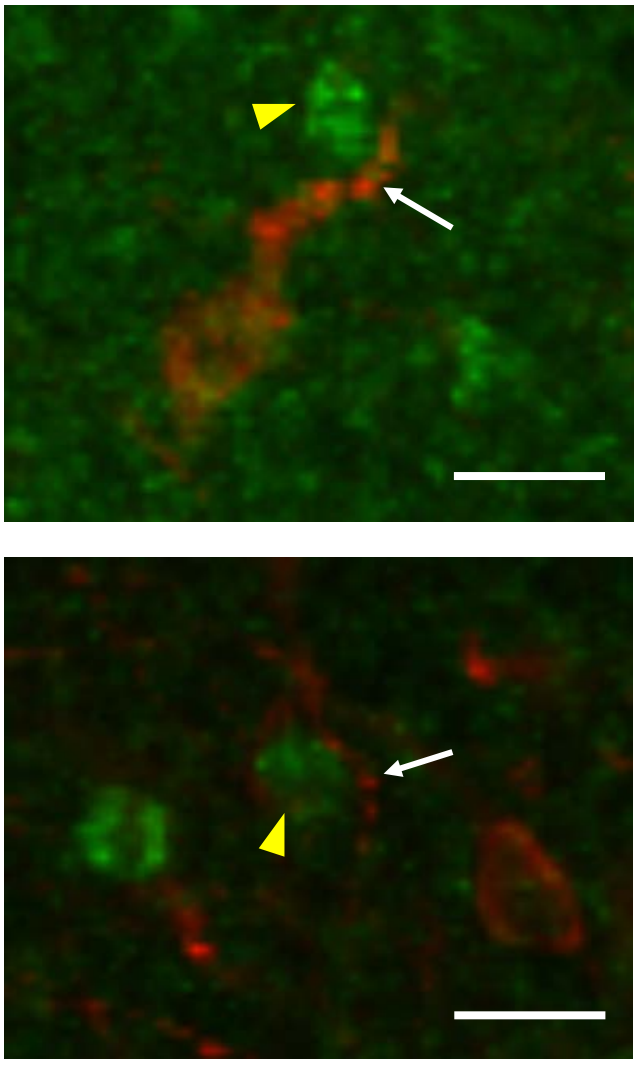


Figure 2-4. Representative photo for the projection from kisspeptin neurons to *Oprk1*-expressing cells in the ARC. The green stained cells marked by yellow arrowheads are *Oprk1*-expressing cells. White arrows marked axons containing kisspeptin immunofluorescence that located close to *Oprk1*-expressing cells. Scale bars: 20 μm.

CHAPTER 3

Investigation of the role of *Oprk1*-expressing cells in the ARC in regulating pulsatile LH secretion

Introduction

It has been reported that *Oprk1* is co-expressed in the KNDy neuron: KNDy neurons might release and receive NKB both as a start signal of kisspeptin section, and Dyn, as a cease signal to cause exponential decrease of GnRH/LH in the autocrine or paracrine ways [61,63,64]. Thus, KNDy neurons might own the ability to generate rhythmic oscillations.

On the contrary, the experiments in Chapter 2 demonstrated that *Oprk1*-expressing cells are different from KNDy neurons in the ARC. This result proposed the existence of another neuronal circuit mediating the Dyn-KOR signaling in control of kisspeptin release. Since little is known about the *Oprk1*-expressing cells, it is of great importance to clarify 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 [42, 47,53], we could still need more knowledge about the neural pathway within and downstream to *Oprk1*-expressing cells.

In the present Chapter, I took use of a cytotoxin to investigate the function of *Oprk1*-expressing cells in regulating the pulse generation. Saporin is a ribosome-inactivating protein that can kill cells by preventing protein synthesis [65], but lacks the specificity and the ability to enter live cells. When saporin is conjugated to specific ligand, the conjugate can bind to specific receptors to enter and kill the cells [66,67].

Previous study showed that rats receiving intra-ARC injection of NKB-SAP showed a depletion of NK3R positive cells, while the number of ARC POMC neurons remained unchanged [68]. This indicates that NKB-conjugated saporin selectively destroyed NK3R-expressing cells. In the present experiment, Dyn-conjugated-saporin (Dyn-SAP) was locally injected into the ARC of adult female rats to destroy *Oprk1*-

expressing cells. By examining the effects of ablation of *Oprk1*-expressing cells on pulsatile LH secretion, the current experiments tried to clarify the role of Dyn-KOR signaling in regulating GnRH pulse generation.

Materials & Methods

Cell culture

P19C6 carcinoma cells (origin) or L6 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, ATCC, Manassas, VA, USA) supplemented with 15% heat inactivated fetal bovine serum (FBS, Sigma, St. Louis, MO, USA). Antibiotic-Antimycotic (Thermo Fisher Scientific, USA), containing 10,000 U penicillin, 10,000 µg streptomycin and 25 µg amphotericin in 1ml was added to the culture medium with a final concentration of 1%. The cell cultures were incubated at 37 °C in a 5% CO_2 humidified atmosphere.

RNA extraction and reverse transcription PCR

Total RNA from P19C6 cells and L6 cells were isolated by chloroform extraction using ISOGEN reagents (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Total RNA (500 ng) from each RNA sample was used for reverse transcription using ReverTra Ace qPCR RT Master Mix (FSQ-301, TOYOBO CO., LTD, Tokyo, Japan) under following conditions: 37 °C for 15 min and 50 °C for 5 min. Integrity and purity of cDNA were assessed using spectrophotometer NanoDrop-1000 (Thermo Fisher Scientific488, USA). cDNA samples were then assigned to PCR using a pair of primers (5'-TTTGTGGTGGGCTTAGTGGG-3', 5'-TGCCAGTAGCCAAATGCAGA-3', product length: 351 bp) targeting rat *Oprk1* gene. The reactions were carried out facilitating ProFlex 3×32 thermal cycler (PE Applied Biosystems, CA, USA) with the following conditions: denaturation at 95 °C for 5 min, followed by PCR amplification (35 cycles) of 95 °C for 30 s, 60 °C for 30 s, and extension at 72 °C for 30 s. The reactions were terminated after extra 10-min

incubation at 72 °C to allow for extension of PCR products. Negative control PCR was run with the template DNA replaced by UPW. PCR products were checked using agarose gel electrophoresis.

Cell viability assay with MTS test

For the investigation of SAP and Dyn-SAP's effect on cells with *Oprk1* expression, P19C6 cells were seeded at 1,980 cells/well in 96 well plates and cultured in 100 µl of culture medium described above. Saporin or Dyn-SAP (Advanced Targeting Systems, San Diego, CA) were added to the medium to reach a final concentration of 10^{-8} M. A third group of cells received the same volume of PBS and were used as reference of viability. Each group have three wells in one plate, and the experiment was carried out in triplicate (three plates in total, 9 wells for each group). After 72 hours of incubation, the cytotoxic effect of Dyn-SAP on the cell viability was evaluated using the CellTiter 96 ®Aqueous One Solution Cell Proliferation Assay (Promega, WI, USA). For this test, 20 µl of reagent containing a tetrazolium compound (3-(4,5-dimethylthiazol-2yl)-5-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) was added to each well and the cells were incubated at 37°C for 1 h. The absorbance of each well was measured in a spectrophotometric multimode plate reader (PelkinElmer, MA, USA). For the investigation of SAP and Dyn-SAP's effect on cells with *Oprk1* expression, the same experiments were carried out using L6 cell line. The results are expressed as percentage of the absorbance of saporin or Dyn-SAP treated cells relative to the PBS added cells.

Animals and treatment

Young adult, female Wistar-Imamichi rats (8 weeks old at the time of brain surgery, 11 weeks old, 200–250 g at the time of blood sampling, Institute for Animal Reproduction, Ibaraki, Japan,) were individually housed in a quiet, temperature (22 ± 2 °C) and humidity-controlled room in the University of Tokyo with a 14-h light, 10-h dark cycle (lights on at 0500 h). Rats had ad libitum access to water and food (CE-2; Clea Japan Inc., Tokyo, Japan). Two normal estrous cycles were confirmed by vaginal smear before brain surgeries. Two weeks after the brain surgery, rats were bilaterally ovariectomized (OVX) and implanted with silastic tubing (i.d., 1.57 mm; o.d., 3.18 mm; 25 mm in length; Dow Corning, Midland, MI, USA) filled with estradiol (E2) (Sigma, St. Louis, MO, USA, 20 μg/ml peanut oil) containing peanuts oil to serve as the diestrus model [69]. Schedule of all the steps in this study was summarized as in Fig. 3-1. Surgeries, including the brain surgery and intravenous cannulation described in the following, were performed under isoflurane anesthesia. All rats were injected with antibiotics (MycillinSl; Meiji Seika, Tokyo, Japan) after any surgery. All protocols were conducted under the “Guidelines for Proper Conduct of Animal Experiments” from the University of Tokyo.

Brain surgeries

Rats were divided into two groups to receive the injection of Dyn-SAP or saporin (Advanced Targeting Systems, San Diego, CA) into the ARC using the Stereotaxic device (Narishige, Tokyo, Japan). When fixing rat head to Stereotaxic device, tooth bar was set to a position to make vertical coordinate of bregma and lambda the same. Injections were made bilaterally (two sites per side) to target the ARC. The anterior sites were 2.5 mm posterior to bregma, ± 0.5 mm lateral, and 9.8 mm ventral to skull

surface. The posterior injections were made 3.6 mm posterior to bregma, ± 0.5 mm lateral, and 10.1 mm ventral to skull surface according to Mittelman-Smith et al [68] and our previous experiences.

A microsyringe pump (EICOM, Kyoto, Japan) with a 25 μl microsyringe (702N, HAMILTON, Reno, NV, USA) was used to achieve the microinjections at a rate of 100 nl/min for 2 min. The needle was left in place for another 5 minutes to adequate diffusion of the drug before removal. Both Dyn-SAP and saporin were diluted with 0.01M PBS to a final concentration of 20 ng/200 nl for Dyn-SAP or 18.6 ng/200 nl for saporin. Animals receiving the infusion of saporin were used as control group. The concentration of unconjugated saporin was decided to be equivalent to saporin present in the Dyn-SAP as described in the manufacturer's product information. The amounts of Dyn-SAP (20 ng) given to each injection site was decided according to previous reports using other kinds of ligand-conjugated saporin [68].

Intravenous cannulation and serial blood sampling

6 days after OVX surgery, a silicon tubing (i.d., 0.5 mm; o.d., 1.0 mm; Shin-Etsu Polymer Co., Ltd, Tokyo, Japan) was inserted into the right atrial. It was 28 mm inside the vein with an extension part going around the neck hypodermically and finally took out from an incision on the back. The cannula was kept in a plastic case affixed to the dorsal skin of the neck until blood sampling. This surgery was done under the instruction of a previous report by Tsukamura H. et al [70].

One hour before the beginning of blood sampling, rats were moved into the blood sampling case. Blood samples (100 μl) were collected every 6 min for 3 h from 1300 h using 1-ml syringes coated with heparin. An equivalent volume of rat red blood cells

taken from donor Wistar-Imamichi rats washed and diluted by heparinized saline was rendered through the same cannula after each blood sampling. Plasma was separated by centrifugation (15,300g, 20min, 4°C) and stored at –20 °C until LH assay.

Nissl staining and in situ hybridization

Brains were sampled from rats transcardially perfused with 50 ml phosphate buffered saline (PBS; 0.05 M phosphate buffer, 0.1 M NaCl, pH 7.4) followed by 50 ml 4% paraformaldehyde in 0.05M phosphate buffer (PB, pH 7.4). Tissues were post-fixed in the same fixative at 4°C for 16 h, cryoprotected in 30% sucrose in PB for 48 h and frozen in Tissue-Tek (Sakura Finetek, Tokyo, Japan) at -20°C for sectioning. Four series of serial coronal sections at 50 μm thickness were prepared using a cryostat (Leica CM1860, Heidelberg, Germany) and conserved in cryoprotectant (0.5 M sucrose, 6.4 mM $MgCl_2$, 0.1 M NaCl, 0.05 M PB, 50% glycerol, pH 7.4) at -30 °C before use.

For Nissl staining, coronal brain sections from both saporin-administrated group and Dyn-SAP-administrated group were stained with thionin solution. Thionin-stained brain sections from every individual underwent microscopic inspection (DM 2500, Leica Microsystems, Wetzlar, Germany) for confirmation of successful of the site of intra-ARC injection of saporin or Dyn-SAP, and only data from subjects in which brain surgery was verified to be successful were subjected to other analyses.

For the confirmation of ablation of KOR containing cells and investigation of the *Kiss1* neuron number in the ARC, *Oprk1* and *Kiss1* mRNA was detected by free-floating in situ hybridization using the DIG-labeled antisense cRNA probe aiming position 264-1404 of NM_001318742 for *Oprk1* or fluorescein-labeled antisense

Kiss1 cRNA probe (position 33-348 of AY196983.1).

Brain sections were treated with 1 μg/ml proteinase K (Takara Bio, Otsu, Japan) in 1ml buffer (1 mM EDTA, 0.1 M NaCl, 10 mM Tris-HCl, pH 7.6) for 20 minutes at 37 ºC to increase permeability. For hybridization, sections were incubated with cRNA probes at a concentration of 1μg/ml in hybridization-buffer containing 50% formamide and 4% dextran sulfate for 16 h at 60 °C. After hybridization, sections were rinsed in 4× SSC containing 50% formamide. Sections were then treated with 20 μg RNase A (Roche Diagnostics, Mannheim, Germany) diluted in 1 ml TNE buffer for 30 min at 37 ºC. The RNase was incubated at 80 ºC for 30 min to inactivate DNase as instructed in the product's homepage. Sections were rinsed in 2 × SSC followed by 0.5 × SSC. Brain sections for the staining of *Oprk1* mRNA were then incubated with alkaline phosphatase conjugated anti-DIG (anti-DIG-AP) antibody (Roche Diagnostics, Mannheim, Germany) at a dilution of 1:1000 in 1 ml blocking reagent (Roche Diagnostics) for 16 h at room temperature. On the third day of ISH, 2 ml NBT/BCIP (Roche Diagnostics) diluted in AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl) was added to the tube for visualizing. Mg^{2+} was not included in this buffer according to the manufacture's instruction. After 20 hours of color development in a dark condition, sections were mounted on slide glasses and bleached with 100 % EtOH for 5 minutes to low the background. Slides were treated with xylene for 15 minutes and then sealed with Entellan New rapid mounting medium (Merck, Darmstadt, Germany). Sections incubated with rKiss1 antisense cRNA probe were incubated with ANTIFLUORESCEIN-HRP CONJUUGATE (anti-FITC-HRP, PerkinElmer, Boston, USA) at a dilution of 1:500 in 1 ml blocking reagent for 1.5 h at room temperature. TSA biotin detection kit (PerkinElmer, Boston, USA) was used

to bring biotin into this system, followed by 1.5 h incubation with Streptavidin Alexa Fluor 488 conjugate (Invitrogen, Eugene, USA) at a dilution of 1:200 for coloration.

The number of *Oprk1*-expressing cells and kisspeptin neurons was counted for every section (200 μm apart) through the ARC, paraventricular hypothalamic nucleus (PVN) and ventromedial nucleus of the hypothalamus (VMH). Cells expressing *Oprk1* mRNA were identified using a bright-field microscope (Olympus BX51, Tokyo, Japan) by two skilled members of our lab without knowing the treatment for each slide. Images of kisspeptin neurons were taken by LSM 700 laser scanning microscope (Carl Zeiss, Inc. Thornwood, USA). Number of kisspeptin neurons was counted using ImageJ.

Radioimmunoassay (RIA) for LH

Plasma LH concentration was determined by a double antibody radioimmunoassay (RIA) with a rat LH RIA kit provided by the National Hormone and Peptide Programme (Baltimore, MD, USA) and were expressed in terms of NIDDK rat LH RP-3. The least detectable level of LH assay was 0.16 ng/ml for 50 μl plasma. The intra-assay was 6.81% at 3.29 ng/ml. All the samples were measured in one assay so there was no inter assay variation.

Statistical analysis

Luteinizing hormone pulses were identified using the PULSAR computer program [71]. The statistical differences in LH pulse parameters and the number of *Oprk1*-expressing cells between Dyn-SAP-treated group and control group were determined by the Student's *t*-test.

Results

Effects of Dyn-SAP on viability of P19C6 cells

In the PCR product amplified from P19C6 cDNA, a band with the proper size of about 351 bps (Fig. 3-2A, left lane) was detected by agarose gel electrophoresis. No other unspecific band was seen in the same PCR product. There was no band detected in PCR product amplified from L6 cDNA (Fig. 3-2A, middle lane) or the negative control PCR (Fig. 3-2A, right lane).

After 72 h of incubation, the relative viability of P19C6 cells with 10-8 M Dyn-SAP was 54.6 ± 1.2 % (mean ± SEM) of cells with PBS. This cell viability was significantly lower than that of P19C6 cells cultured with 10-8 M SAP (73.3 ± 6.2 %, $P < 0.01$, Fig. 3-2B left). The relative viability of L6 cells added with SAP or Dyn-SAP showed no significant difference (Fig. 3-2B right).

Effects of arcuate Dyn-SAP injections on the number of Oprk1-expressing cells in the ARC

Traces of the intra-ARC injection were verified with Nissl-stained brain sections. All animals in both groups appear to have a similar site of injection. The end of injection traces were located at or just above the anterior or posterior part of the ARC, as shown in the representative pictures in Fig. 3-3A. The anteroposterior position was at a range of 2.1 mm- 2.6 mm posterior to bregma for the two anterior sites and 3.4 mm- 3.8 mm posterior to bregma for the two posterior sites. Data from all the individuals was used for the following analysis. No destruction of brain tissues but the cannula trace was observed.

Morphological appearance of *in situ* hybridization-stained brain sections revealed

a loss of *Oprk1*-expressing cells in Dyn-SAP administrated group (Fig. 3-3B). Statistical analysis confirmed a significant decrease of *Oprk1*-expressing cells: the number of the cells was 757 ± 54 in saporin-administrated group and 364 ± 58 in Dyn-SAP administrated-group ($p < 0.01$, Fig. 3-3C).

Effects of arcuate Dyn-SAP injections on the number of Oprk1-expressing cells in neural nucleus other than ARC

In situ hybridization-stained brain sections revealed the similar morphological characteristics of *Oprk1*-expressing cells in both SAP- and Dyn-SAP-administrated group in PVN and VMH (Fig. 3-4A). Statistical analysis, however, showed no significant decrease of *Oprk1*-expressing cell number in PVN (SAP 821 ± 44 vs. Dyn-SAP 769 ± 56) or VMH (SAP 389 ± 30 vs. Dyn-SAP 336 ± 66) (Fig. 3-4B).

Effects of ARC-Oprk1-expressing cell loss on pulsatile LH secretion

Fig. 3-5A shows the representative LH pulses of saporin and Dyn-SAP injected rats. Statistical analysis revealed a significant increase of LH pulse frequency (number of LH pulses in 3 hours) in Dyn-SAP-injected group (SAP 6.50 ± 0.43, $n = 6$ vs. Dyn-SAP 8.43 ± 0.30, $n = 7$; $p < 0.01$; Fig. 3-5B) compared to the control group. Inter-pulse intervals (SAP 29.08 ± 1.44 min vs. Dyn-SAP 21.94 ± 0.96 min) showed significant difference between the two groups ($p < 0.01$). No significant difference was found in mean plasma LH level (SAP 1.47 ± 0.11 ng/ml vs. Dyn-SAP 1.70 ± 0.04 ng/ml, $p = 0.056$) or average amplitude of LH pulses (SAP 1.59 ± 0.11 ng/ml vs. Dyn-SAP 1.33 ± 0.08 ng/ml, $p = 0.069$), but the baseline of LH concentration significantly ($p < 0.05$) increased in Dyn-SAP injected group (1.12 ± 0.06 ng/ml) compared to SAP

injected group (0.87 ± 0.09 ng/ml).

Effects of arcuate Dyn-SAP injections on the number of kisspeptin neurons in the ARC

Kiss1-expressing cells were detected in the ARC of both Dyn-SAP- and saporin-treated animals (Fig. 3-6A). Statistical analysis revealed no significant difference in the number of *kiss1*-expressing cells: the number of the cells was 1576 ± 67 in saporin-administrated group and 1475 ± 64 in Dyn-SAP administrated-group (Fig. 3-6B).

Discussion

The experiments in this Chapter demonstrated that *Oprk1*-expressing cells in the ARC is involved in inhibiting LH pulse generation, because the chemical lesion of *Oprk1*-expressing cells caused an increase in LH pulse frequency and the baseline of plasma LH concentration. Both *in vitro* and *in vivo* Dyn-SAP administration resulted in a significant decrease in the number of *Oprk1*-expressing cells compared to the SAP-treated control group. This suggests not only the effectiveness of Dyn-SAP on cells expressing *Oprk1*, but also the nontoxicity of saporin for any cells under the current experimental condition. Nether SAP or Dyn-SAP decreased relative viability of L6 cells, which demonstrated that the toxicity of Dyn-SAP is mediated by KOR. Besides, the number of kisspeptin neurons showed no significant difference between Dyn-SAP- and SAP-treated groups, suggesting that Dyn-SAP had no influence on cells other than *Oprk1*-expressing ones. Taken together, the increase in LH frequency and baseline concentration in Dyn-SAP administrated group was thought to be a result of the lesion of ARC *Oprk1*-expressing cells.

Previous studies using KOR agonists or antagonists demonstrated the suppressing effect of Dyn on GnRH/LH secretion [42, 47,53]. In our study, we considered what the signaling pathway mediating this influence is. It has widely been accepted that KNDy neurons contain KOR to receive the Dyn signal in a paracrine or autocrine way, although the evidence is only based a previous radio-ISH study [61]. According to the present results, the number of *Kiss1*-expressing neurons did not significantly decrease in Dyn-SAP administrated group as *Oprk1*-expressing cells. This suggests, at least in part, that *Oprk1* is not expressed in most of the kisspeptin neurons. From the results that lesion of *Oprk1*-expressing cells in the ARC can accelerate pulsatile LH secretion, it is concluded that the output of *Oprk1*-expressing cells to KNDy neurons might be

a “negative signal” for the activity of KNDy neurons. In our experiments, when *Oprk1*-expressing cells in the ARC were ablated by the injection of Dyn-SAP, this “negative signal” was also damaged and resulted in accelerated LH pulses.

In conclusion, the experiments in this Chapter demonstrated the inhibitory role of *Oprk1*-expressing cells in the ARC in regulating LH pulse generation. These results also brought another issue on how the signal pathway inside Dyn-KOR signaling is to promote *Oprk1*-expressing cell activity and releasing of the signal that suppress kisspeptin secretion from KNDy neurons. More details will be investigated for solid evidence for this signaling pathway from *Oprk1*-expressing cells to KNDy neurons. Finally, the results considering kisspeptin neuron number showed that *Oprk1*-expressing cells are different from kisspeptin neurons.

Figures

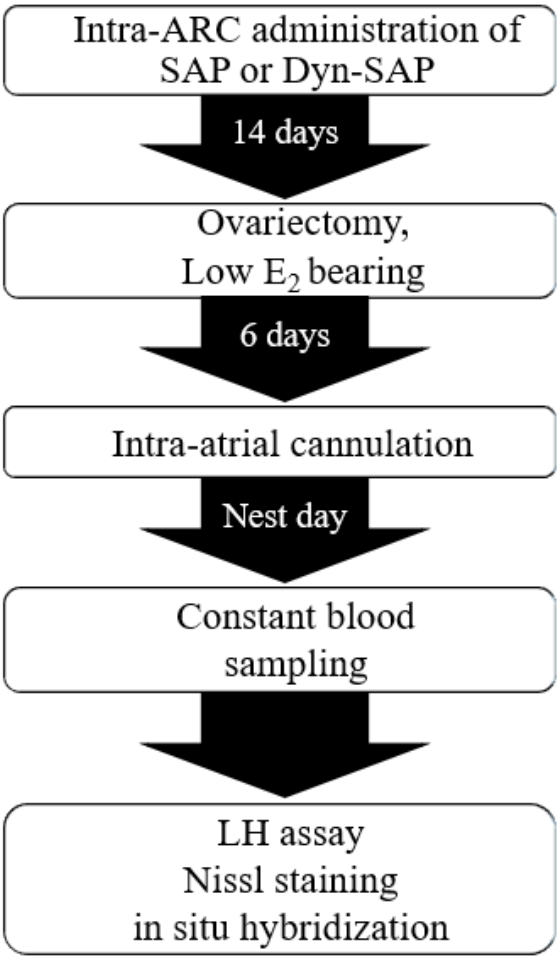
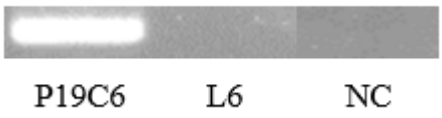


Figure 3-1. Summary of protocol steps in the *in vivo* experiment. SAP or Dyn-SAP was injected into ARC by brain surgeries. Four points of injection aiming to ARC were made. Two weeks after brain surgery, rats received ovariectomy with low estradiol bearing to make a diestrous model. 6 days later, rats received intra-atrial cannulation. Constant blood sampling was carried out the next day from the cannula and brain samples were collected after the blood sampling.

A



B

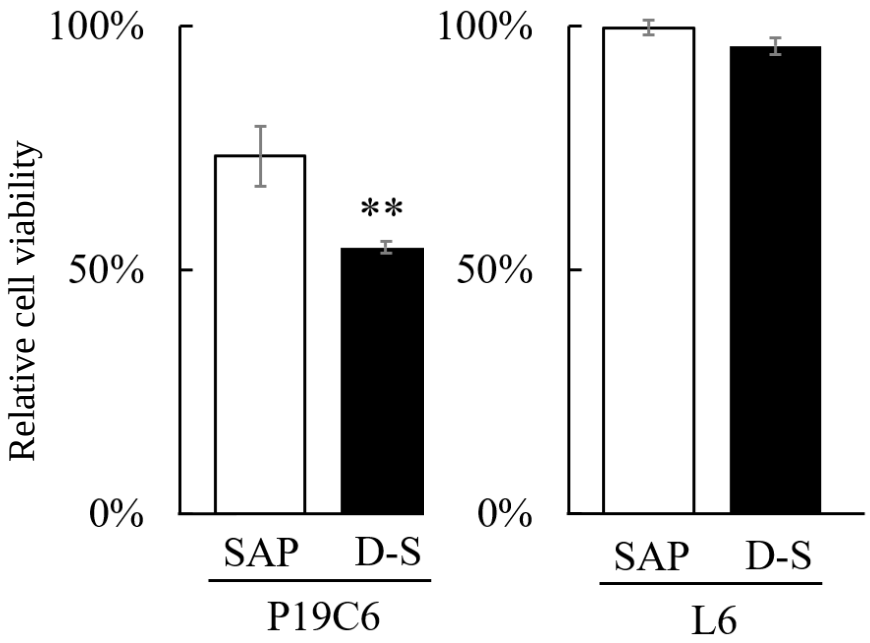


Figure 3-2. The effects of Dyn-SAP on viability of P19C6 and L6 cell lines. **A**: *Oprk1* band (351 bp) from the PCR product carried out with cDNA from P19C6 and L6 cells as template. NC, negative control. **B**: left, relative cell viability of P19C6 cells incubated with saporin (SAP) or Dyn-SAP compared to cells incubated with vehicle; right, relative cell viability of L6 cells incubated with SAP or Dyn-SAP compared to those incubated with vehicle. Values are means ± SEM. ** $P < 0.01$, Student's *t*-test.

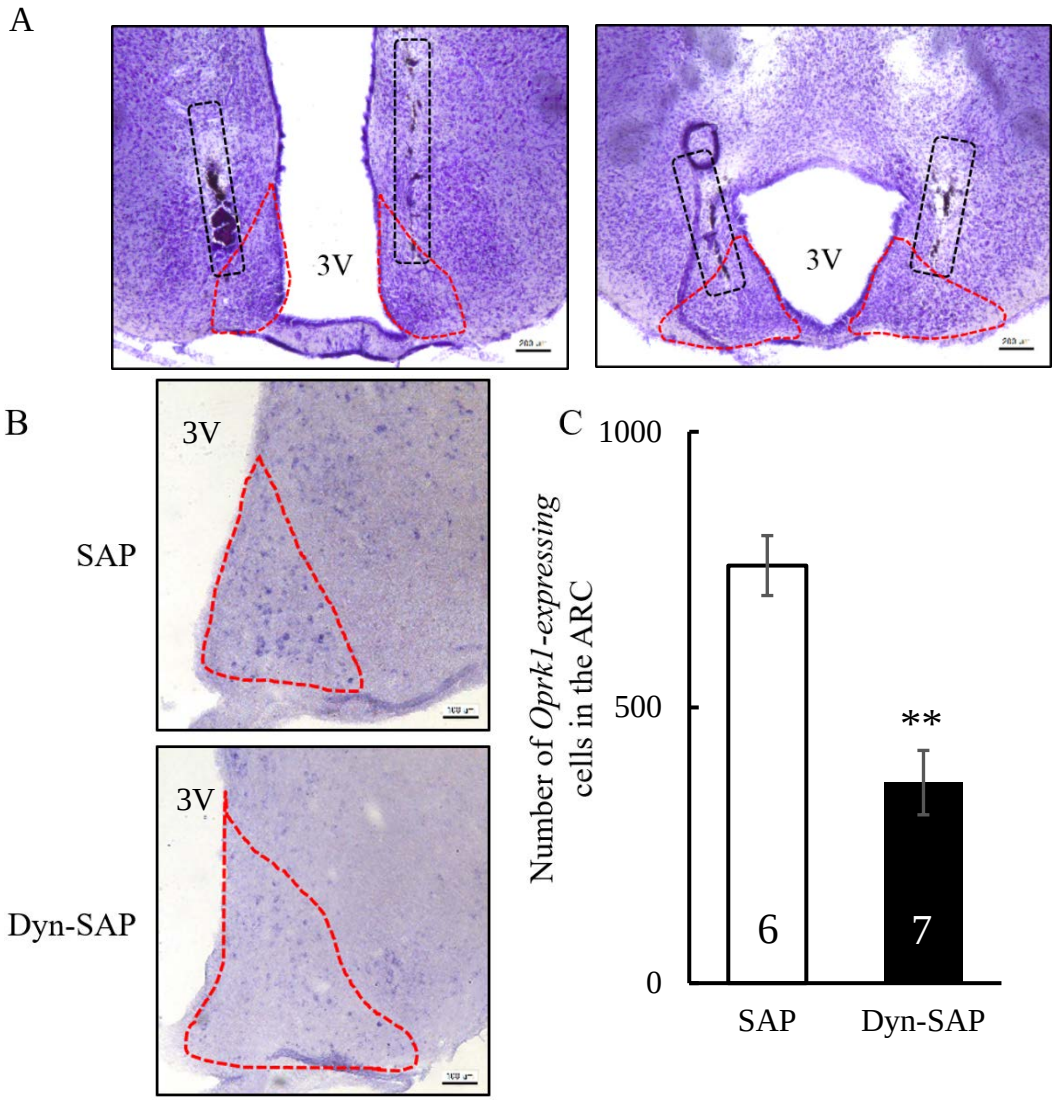


Figure 3-3. The effects of Dyn-SAP injection on *Oprk1* expression in the arcuate nucleus (ARC). **A**: Verification of the injection locations in anterior and posterior ARC (Scale bars: 200 μm). Black dotted lines show the injury of cannula and red dotted lines show the area of the ARC. 3V, third ventricle. **B**: representative photomicrograph of *Oprk1*-expressing cells stained by *in situ* hybridization in SAP group or Dyn-SAP group. The ARC was circled with red dotted lines (Scale bars: 100 μm). **C**: The number of *Oprk1*-expressing cells in the ARC of SA- or Dyn-SAP-treated group. Values are means ± SEM. ** $P < 0.01$, Student's *t*-test.

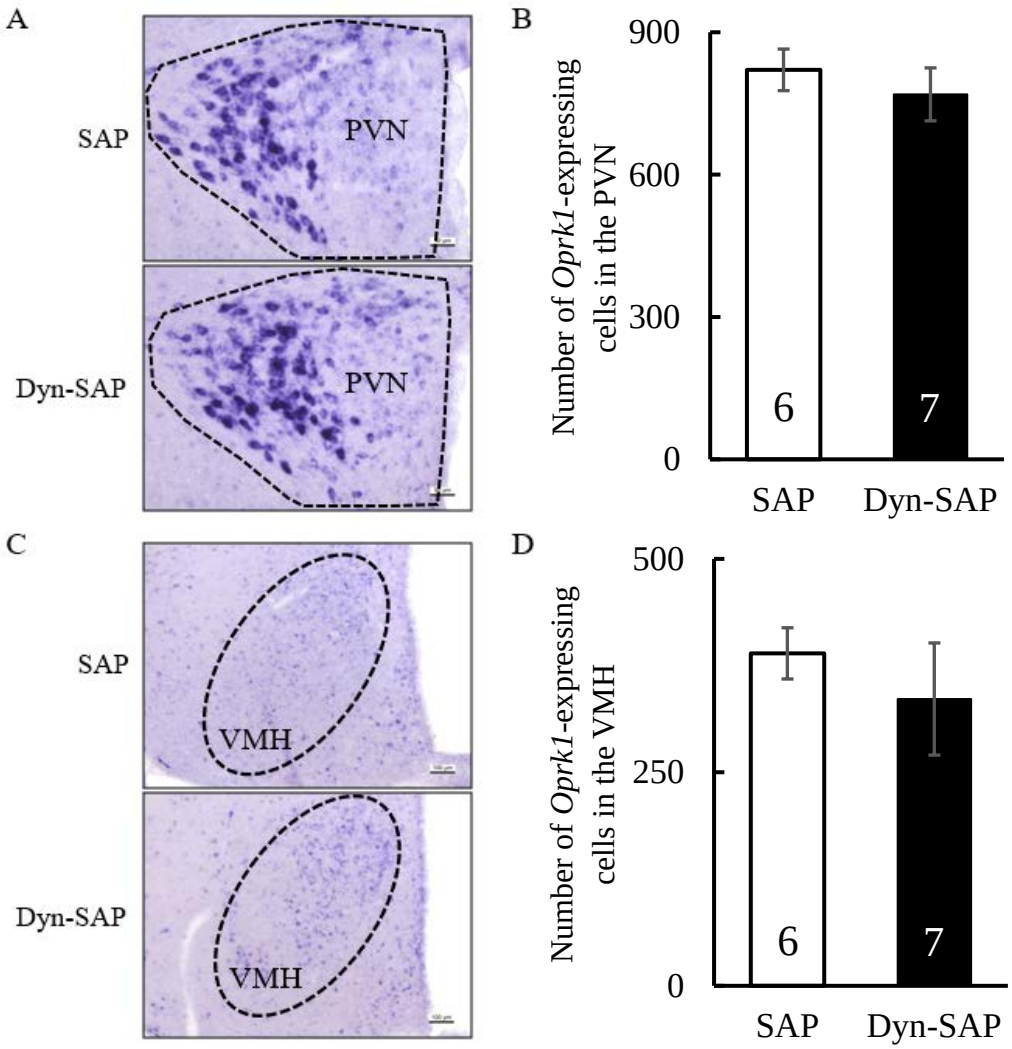
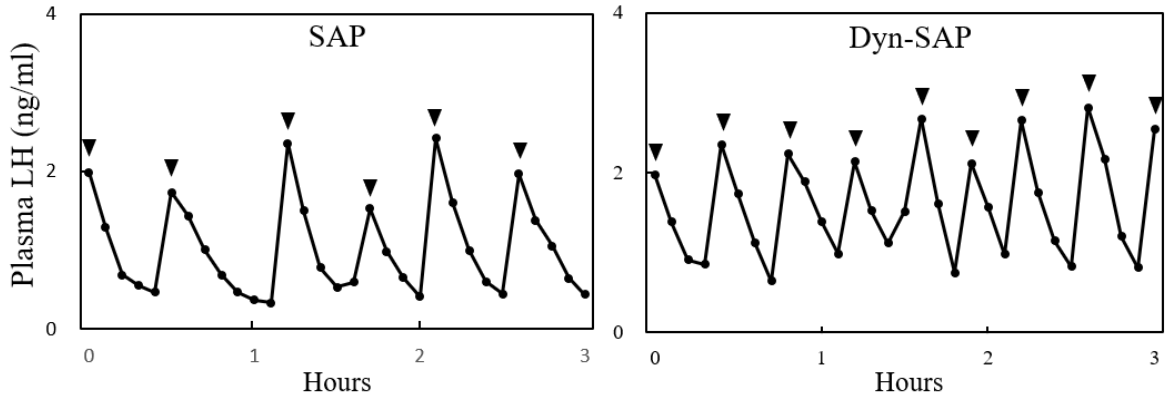


Figure 3-4. The effects of arcuate Dyn-SAP injection on *Oprk1* expression in the PVN and VMH. **A**: Representative photomicrograph of *Oprk1*-expressing cells in the PVN stained by *in situ* hybridization in SAP group or Dyn-SAP group. The PVN was circled with black dotted lines (Scale bars: 50 μm). **B**: The number of *Oprk1*-expressing cells in the PVN of SAP- or Dyn-SAP-treated group. Values are means ± SEM. The numbers in each column represent the numbers of animals used in each group. **C**: Representative photomicrograph of *Oprk1*-expressing cells in the VMH. The VMH was circled with black dotted lines (Scale bars: 100 μm). **D**: The number of *Oprk1*-expressing cells in the VMH of saporin- or Dyn-SAP-treated group. Values are means ± SEM.

A



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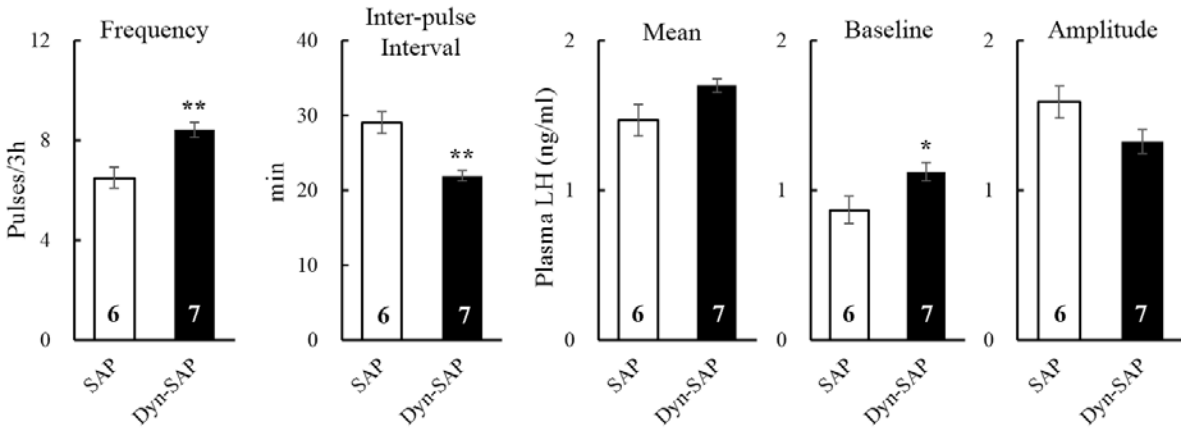

Figure 3-5. Effects of arcuate Dyn-SAP injection on pulsatile LH secretion. **A**: profiles of LH release in representative rats injected with SAP or Dyn-SAP. Blood samples were collected for 3 h at 6-min intervals. Arrowheads indicate the peaks of LH pulses identified by the PULSAR computer program. **B**: frequency of LH pulses, LH pulses interval, and mean, baseline, amplitude of plasma LH concentrations in saporin or Dyn-SAP injected group. Values are means ± SEM. The numbers in each column represent the numbers of animals used in each group. ** $P < 0.01$, * $P < 0.05$, Student's *t*-test.

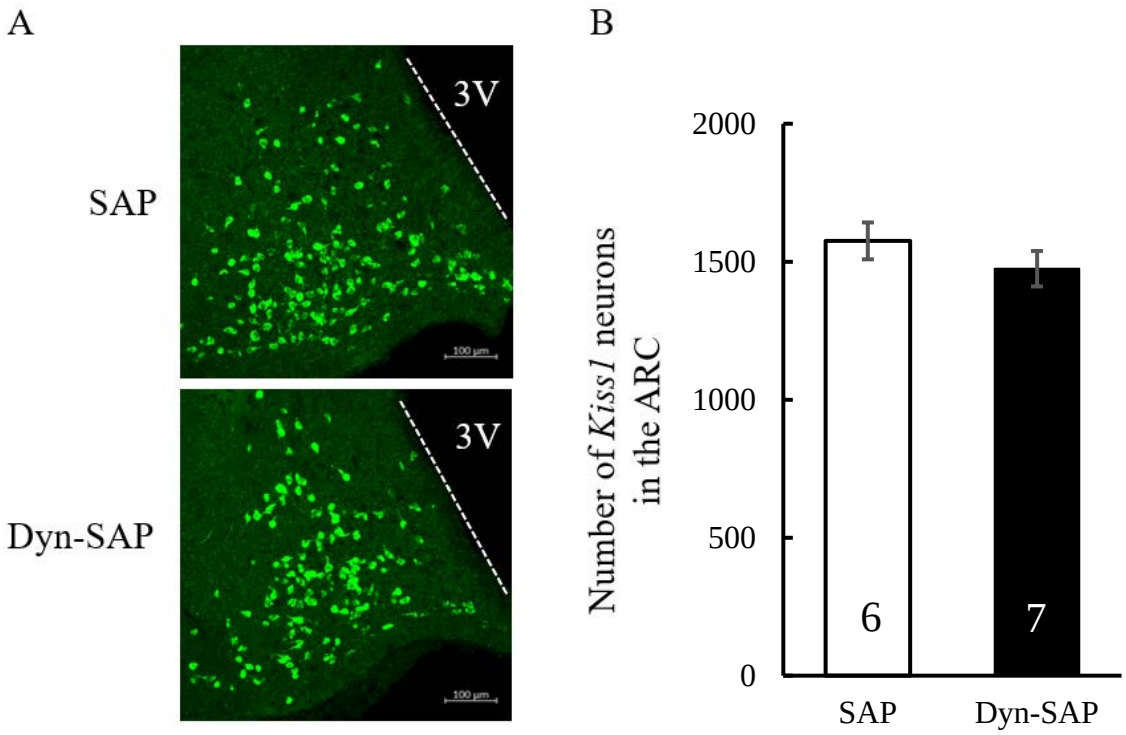

Figure 3-6. *In situ* hybridization of r*Kiss1* in the ARC. A, representative ISH photos of rKiss1 from rats injected with SAP (top) or Dyn-SAP (bottom). White dotted lines show the edge of third ventricle. 3V, third ventricle. Scale bars: 100 μm. Cell counting of kisspeptin neuron in the ARC was determined using the ImageJ software (B). Values are means ± SEM. The numbers in each column represent the numbers of animals used in each group.

CHAPTER 4

Response of *Oprk1*-expressing cells to Dyn stimulation

Introduction

KOR is one of the G protein-coupled receptors (GPCRs). Receptors in this family are associated with G proteins, which consist of alpha (α), beta (β) and gamma (γ) subunits [72]. The β and γ subunits can form a stable dimeric complex to bind to the cytomembrane. When GPCRs bind to the ligands, they activate an associated G protein by exchanging the GDP bound to the G protein for a GTP. The α subunit will then dissociate from β and γ subunits for the further intracellular signaling cascades [72].

The G protein coupled with KOR has the Gi type α subunit. Most of the Gi proteins are known for their suppressive effect to decrease cell activity. It is reasonable to infer from these reports that *Oprk1*-expressing cells in the ARC stop to release signals when Dyn is bond to KOR. This speculation, however, is inconsistent with the result in the Chapter 3. As is concluded from the results in the last chapter, signals released from *Oprk1*-expressing cells to KNDy neurons are supposed to be a “negative signal” because lesion of *Oprk1*-expressing cells resulted in an increase of the LH pulse frequency and baseline of plasma LH concentration. If Dyn actually inactivates *Oprk1*-expressing cells, Dyn administration would suppress the secretion of this “negative signal” and promote LH secretion but not stop it. Hence, it is needed to have some ideas about the circuit among Dyn-KOR signaling.

The experiments in the present Chapter aim to clarity the response of *Oprk1*-expressing cells to Dyn. Nalfurafine (Toray, Japan), an efficient KOR agonist [73], was injected into the third ventricle of adult female rats to stimulate the KOR-expressing cells. The activation of KOR cells was monitored by c-Fos staining.

Materials and Methods

Animals and treatment

Young adult, female Wistar-Imamichi rats (8 weeks old at the time of brain surgery and ovariectomy, Institute for Animal Reproduction, Ibaraki, Japan) were individually housed in a quiet, temperature- (22 ± 2 °C) and humidity-controlled room with a 14-h light, 10-h dark cycle (lights on at 0500 h). Rats had ad libitum access to water and food (CE-2; Clea Japan Inc., Tokyo, Japan). Two normal estrous cycles were confirmed by vaginal smears before brain surgeries and bilateral ovariectomy. A Silastic tubing (i.d., 1.57 mm; o.d., 3.18 mm; 25 mm in length; Dow Corning, Midland, MI, USA) filled with estradiol (E2) (Sigma, St. Louis, MO, USA, 20 μg/ml peanut oil) containing peanuts oil was subcutaneously implanted in each animal to serve as the diestrus model [69]. All the surgeries, including the brain surgery and intravenous cannulation were performed under isoflurane anesthesia. All rats were injected with antibiotics (Mycillin; Meiji Seika, Tokyo, Japan) after any surgery. All protocols were conducted under the “Guidelines for Proper Conduct of Animal Experiments” from the University of Tokyo.

Brain surgeries

Rats were implanted with a stainless-steel guide cannula (22 G, Plastics One, Roanoke, VA, USA) directed towards the third cerebroventricle (3V) by stereotaxic surgery. The stereotaxic coordinates for implantation of guide cannula were 0.8 mm posterior and 7.5 mm ventral to the bregma at the midline according to the rat brain atlas [74]. The guide cannula was fixed to the skull using dental cement (Quick Resin; Matsukaze, Kyoto, Japan). The rats were allowed a one-week recovery period prior

to drug administration.

Drug administration

Nalfurafine was dissolved in artificial cerebrospinal fluid (aCSF) at a final concentration of 1 mg/ml. Nalfurafine solution (5 nmol/head) was infused into the 3V at a flow rate of 0.939 μl/min for 3 min using a microsyringe pump (Eicom, Kyoto, Japan) through an inner cannula (28 G, Plastics One), which was inserted into the guide cannula. The needle was left in place for another 5 minutes to adequate diffusion of the drug after the infusion. Since there was no previous report infusing nalfurafine into 3V, the amount of nalfurafine was decided according to other studies injecting nalfurafine subcutaneously or intravenously. Rats in the control group were infused with the same volume of vehicle (aCSF).

***Nissl staining and double staining of Oprk1 and* c-Fos**

One hour after the drug administration, brains were collected from rats that were transcardially perfused with 50 ml phosphate buffered saline (PBS; 0.05 M phosphate buffer, 0.1 M NaCl, pH 7.4) followed by 50 ml 4% paraformaldehyde in 0.05M phosphate buffer (PB, pH 7.4). Tissues were post-fixed in the same fixative at 4°C for 16 h, cryoprotected in 30% sucrose in PB for 48 h and frozen in Tissue-Tek (Sakura Finetek, Tokyo, Japan) at -20°C for sectioning. Four series of serial coronal sections at 50-μm thickness were prepared using a cryostat (Leica CM1860, Heidelberg, Germany) and conserved in cryoprotectant (0.5 M sucrose, 6.4 mM $MgCl_2$, 0.1 M NaCl, 0.05 M PB, 50% glycerol, pH 7.4) at -30 °C before use.

For Nissl staining, coronal brain sections from both aCSF-administrated group and

nalfurafine-administrated group were stained with thionin solution. Thionin-stained brain sections from every individual underwent microscopic inspection (DM 2500, Leica Microsystems, Wetzlar, Germany) for confirmation of the site of i.c.v injection. Only data from subjects in which brain surgery was verified to be successful were subjected to other analyses.

To investigate the response of *Oprk1*-expressing cells in the ARC to KOR agonist, *Oprk1* mRNA and c-Fos protein were dual-stained in the same brain sections by free-floating in situ hybridization using the DIG-labeled antisense cRNA probe aiming position 264-1404 of NM_001318742 and rabbit anti-rat c-Fos antibody (Merck, Darmstadt, Germany), respectively. Brain sections were first washed by PBS, followed by 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature. For hybridization, sections were incubated with cRNA probes at a concentration of 1μg/ml in hybridization-buffer containing 50% formamide and 4% dextran sulfate for 16 h at 60 °C. For the linearization, the probes had been preheated in hybridization-buffer for 3 minutes at 85 ºC and chilled on ice for 5 minutes prior to hybridization.

After hybridization, sections were rinsed in 4× SSC containing 50% formamide. Sections were then treated with 20 μg RNase A (Roche Diagnostics, Mannheim, Germany) diluted in 1 ml TNE buffer for 30 min at 37 ºC. The RNase solution was incubated at 80 ºC for 30 min to inactivate DNase as instructed in the manufacturer's instruction. Sections were rinsed in 2 × SSC followed by 0.5 × SSC and then subjected to blocking with 1 ml of TBS buffer containing 1.0 % Blocking Reagent (Roche Diagnostics). After the blocking, brain sections for the staining of *Oprk1* mRNA were then incubated with alkaline phosphatase conjugated anti-DIG (anti-DIG-AP)

antibody (Roche Diagnostics, Mannheim, Germany) at a dilution of 1:1000 in 1 ml blocking reagent (Roche Diagnostics) for 16 h at room temperature. On the third day of ISH, 2 ml NBT/BCIP (Roche Diagnostics) diluted in AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl) was added to the tube for visualizing. Mg^{2+} was not included in this buffer according to the manufacturer's instruction.

After 20 hours of color development in a dark condition, sections were treated with 1% H_2O_2 in PBS for 10 minutes to remove endogenous peroxidase. Sections were incubated with 10% normal goat serum for 1 h and then with rabbit anti-rat c-Fos antibody (ABE457, Merck, Darmstadt, Germany) for 30 h. After incubated with goat anti-rabbit IgG conjugated to biotin (BA-1000, Vector Laboratories, CA, USA), sections were treated with VECTASTAIN *Elite* ABC kit (Vector Laboratories, CA, USA). Visualization was carried out by DAB-buffer tablets (Merck, Darmstadt, Germany) for 4 min. After mounted on gelatin-coated slides, sections were treated with 100% EtOH for 5min, xylene for 15 minutes and then sealed with Entellan New rapid mounting medium (Merck, Darmstadt, Germany).

The number of *Oprk1*-expressing cells co-expressing c-Fos was counted for every section (200 μm apart) through the ARC using a bright-field microscope (Olympus BX51, Tokyo, Japan).

Statistical analysis

The statistical differences in the number of *Oprk1* and c-fos double stained cells between aCSF and nalfurafine injected groups were determined by Student's *t*-test.

Results

Verification of the trace of i.c.v drug injection

After fixing the cannula into the third ventricle, cerebrospinal fluid has been confirmed during the one week before drug administration and brain sampling. Traces of the i.c.v injection of aCSF or nalfurafine were verified with Nissl-stained brain sections. All animals appear to have a similar site of injection, with the injection trace ended at the 3V as shown in the representative picture in Fig. 4-1. No destruction of brain tissues but the cannula trace pierced from the top of brain into third ventricle was observed.

Effects of Nalfurafine administration on the expression of c-Fos in Oprk1-expressing cells in the ARC

In brain sections from all the individuals in each group, cells express *Oprk1* or c-Fos alone, and cells stained with both *Oprk1* and c-Fos immunoactivity were detected throughout the ARC as shown in representative photos (Fig. 4-2). The number of double-stained cells in the ARC of nalfurafine-administrated group (47.3 ± 6.6) was significantly higher than that of aCSF-administrated group (23.7 ± 1.8) ($p < 0.05$; Fig. 4-3).

Discussion

Results in the present Chapter demonstrated that *Oprk1*-expressing cells were activated by the intracerebroventricular administration of Nalfurafine. Intracerebroventricular (i.c.v) injection through the cannula to the third ventricle has been considered to be a reliable method to administrate drugs into the brain. Confirmation of cerebrospinal fluid ensured that drugs were correctly infused into 3V. Since change of plasma estradiol level can affect *Oprk1* expression [47], animals used in this study received OVX and estradiol tube implantation to become the diestrus model, which is the same model as in chapter 3. Expression of c-Fos is generally used as an indirect marker of neuronal activity because c-Fos is often expressed when neurons are stimulated [75,76]. The present results, therefore, demonstrated that *Oprk1*-expressing cells in the ARC can be activated if combined with the ligand of KOR.

Several intracellular signal pathways have been demonstrated to mediate the effect of Gαi. The most commonly accepted mechanism is the inhibition of adenylate cyclase [77]. In the case of KOR, previous studies demonstrated its core effector to be the G protein-coupled inwardly-rectifying potassium channels (GIRKs) [78,79]. GIRKs is a member of the inwardly rectifying potassium channel (Kir, IRK) family. As indicated by their name, potassium channels in Kir family can be activated by hyperpolarized membrane potential and result in an inward potassium stream, so-called “rectifying effect” [79,80]. Besides regulation by the membrane potential, Kirs are also regulated by G proteins and the upstream GPCR. These two ways of regulation result in opposite direction of potassium current. A famous example of GIRK is a cardiac channel consists of two GIRK1 (Kir 3.1) and two GIRK4 (Kir 3.4)

subunits. This channel is activated by acetylcholine through M2 muscarinic receptors and causes an outward current of potassium, and finally leads to a decrease in heart rate as the cells are hyperpolarized [81]. These conflicting roles of Kirs reminds me of the possibility that GIRK s in different cells may act differently. The GIRK down-stream to KOR is also Kir 3.4. No inspection has been done on the direction of potassium stream conducted by GIRKs in *Oprk1*-expressing cells until this manuscript is written. Therefore, this ability of Kir 3.4 may possibly mediate the activation of *Oprk1*-expressing cells in the ARC in response to KOR agonist injection. As a result of neural firing, c-Fos expression was upregulated.

KOR has also recently been recognized to activate the most kinds of mitogen-activated protein kinases (MAPKs) pathways [82–85]. The MAPK pathway, originally called extracellular signal-regulated kinases, is a series of proteins that transmits a signal from a receptor on the cell surface to the DNA to regulate gene expressions and following cellular events including cell proliferation, differentiation, cell survival and apoptosis. It was proved that extracellular signal-regulated kinase (ERK), known as the classic MAPK, is able to increase the expression of *c-fos* gene [86]. This pathway is also known for its ability to phosphorylate c-Fos to increase its stabilization and activation [87]. More studies have also demonstrated that other classes of MAPK pathways, including p38 mitogen-activated protein kinases (p38 MAPK) and c-Jun N-terminal kinases (JNKs) are in charge of c-Fos activity by regulating phosphorylation of c-Fos or the stability of AP-1, the heterodimer of c-Fos and c-Jun [88,89]. To date, it has been demonstrated that two classes of MAPK, ERK and p38 MAPK pathways can be activated by KOR [82–85]. Although these researches did not discuss the signal pathway from KOR all the way down to c-Fos

and cellular activity, it is reasonable to speculate that MAPK pathways activated by KOR can result in neural activation mediated by c-Fos.

There is also a crosstalk between MAPKs and GIRKs. Besides being activated by M2 muscarinic receptors and causes an outward current of potassium, MAPK pathway provides an alternative reaction to GIRK. The GIRK downstream to KOR is consist of Kir 3.1 and Kir 3.4, in both of which a pair of highly conserved tyrosine residues exist. Tyrosine phosphorylation of these residues inactivates the channel by increasing the rate of Gβγ dissociation, as measured in transfected cells [90,91]. In spinal cord [92,93] and neurons in dorsal raphe nucleus [94], it was also proved that activation of p38 MAPK pathway by KOR stimulates GIRK phosphorylation and results in inhibited conduction of GIRK.

In conclusion, the intracellular signal pathway downstream from Gαi is still under debate. Although it was generally believed that KOR inactivate cells by activating a GIRK, more and more studies have demonstrated that both Gαi and GIRK have multiple reactions depend on the cell type and intracellular pathway. The increase of c-Fos-immunopositive-*Oprk1*-expressing cells in this study demonstrated an increase of cellular activity. This phenomenon can be explained by MAPK-c-Fos signaling or p38 MAPK-GIRK cascade, but more studies are necessary to clarify the exact signaling in *Oprk1*-expressing cells.

Figures

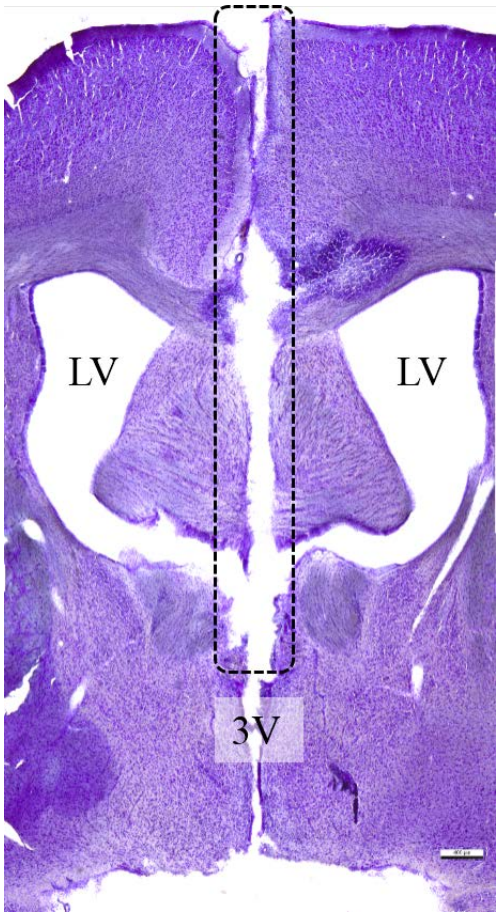

Figure 4-1. Verification of the injection locations in anterior and posterior ARC (Scale bar: 400 μm). Black dotted line shows the trace of i.c.v cannula into 3V. 3V, third ventricle. LV, lateral ventricle.

aCSF

Nalfurafine

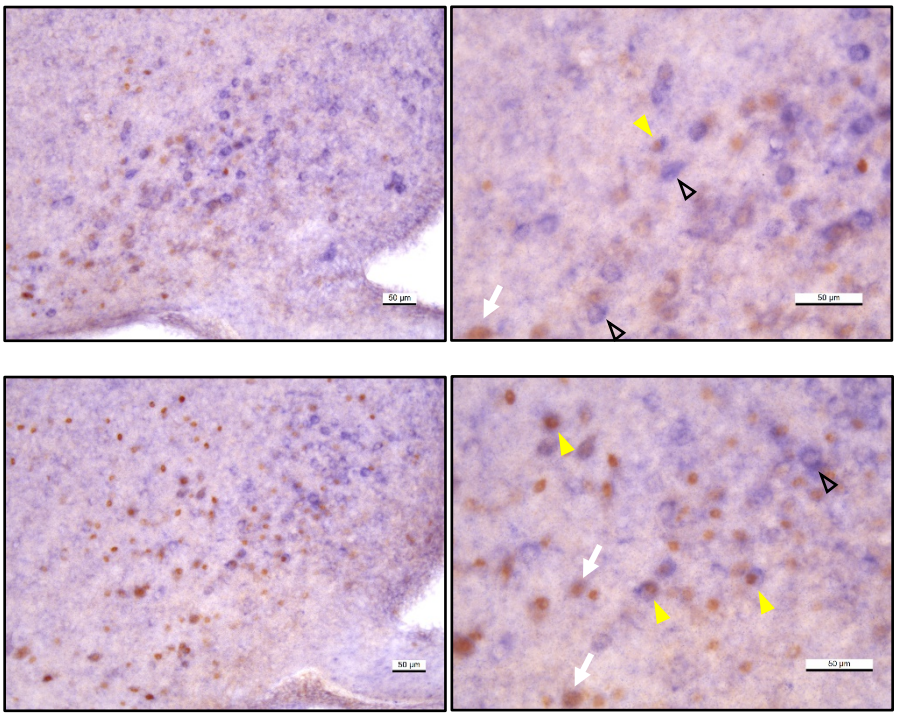

Figure 4-2. Representative photomicrographs for the double staining of *Oprk1* by ISH (purple) and c-Fos by IHC (brown) of coronal sections including the ARC. Photos at the top and bottom panels came from animals injected with aCSF or nalfurafine, respectively. Photos at the right lane show sections at higher magnification. Blank arrowheads mark cells stained by ISH. c-Fos alone stained by IHC in the nucleus are marked by white arrows. Double stained cells are indicated by yellow arrowheads. Scale bars: 50 µm

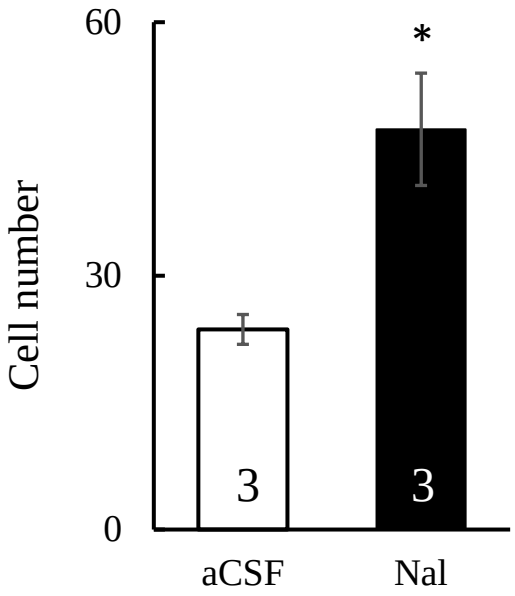

Figure 4-3. Statistical analysis of *Oprk1* and c-Fos double stained cell number in the ARC. Values are means ± SEM. The numbers in each column represent the numbers of animals used in each group. Nal, nalfurafine. (* $p < 0.05$, Student's *t*-test).

CHAPTER 5

General Discussion

Neural circuit between *Oprk1*-expressing cells and KNDy neurons in the ARC

In 2011, Navarro et al reported that kisspeptin neurons in the ARC express *Oprk1* [61]. It was then widely accepted that Dyn secreted from KNDy neurons regulates pulsatile LH secretion in an autocrine or paracrine manner. However, the present dissertation demonstrated that there is no *Oprk1* expression in KNDy neurons. Results of *Oprk1* and kisspeptin double staining in Chapter 2 showed no co-expression (Fig. 2-2). Instead, projections from kisspeptin neurons (some of which were proved to be axons from arcuate-kisspeptin neurons) were found located adjacent to ARC-*Oprk1*-expressing cells, which did not co-express kisspeptin (Fig. 2-4). In addition, the result considering kisspeptin neuron number after injection of Dyn-SAP in Chapter 3 showed that kisspeptin neurons did not decrease together with *Oprk1*-expressing cells (Fig. 3-5). Therefore, it can be concluded as shown in the schematic summary of Fig 5-1 that *Oprk1*-expressing cells in the ARC are different from KNDy neurons. These *Oprk1*-expressing cells receive Dyn input from KNDy neurons then regulate activity of KNDy neurons and pulsatile GnRH/LH secretion in response.

In Chapter 3, Dyn-SAP, a selective *Oprk1*-expressing cell toxin, was used to investigate the role of this newly recognized cell in pulsatile LH secretion. The ablation of *Oprk1*-expressing cells in the ARC caused by the injection of Dyn-SAP resulted in an increase of LH pulse frequency and baseline of plasma LH concentration (Fig. 3-4). It can be referred from the present results that the output of *Oprk1*-expressing cells to KNDy neurons was a “negative signal” to suppress the activity of KNDy neurons. Therefore, when *Oprk1*-expressing cells in the ARC were ablated by the injection of Dyn-SAP, this “negative signal” was also damaged and resulted in accelerated LH pulses.

Currently, whether the signals secreted from *Oprk1*-expressing cells go directly to kisspeptin neurons or control a third kind of cell as an intermediary agent is still not clear. Transgenic rats that can show the downstream cells of *Oprk1*-expressing cells is now under construct. Meanwhile, considering that *Oprk1*-expressing cells express GAD65, the enzyme for the synthesis of γ-aminobutyric acid (GABA) (unpublished results), and the fact that kisspeptin neurons receive GABAergic synaptic input [95], we can hypothesize that *Oprk1*-expressing cells secrete GABA to inhibit kisspeptin neuron activity. But more researches are needed to demonstrate this pathway.

Another question about the Dyn-KOR signaling is its role in the generation of GnRH/LH pulses. Because a number of experiments have shown the suppressing effect of Dyn on LH pulse generation [42,52], it has been speculated that Dyn-KOR signaling works synergistically with NKB-NK3R for the pulse generation. These two signaling that possess opposite effect on neural activity were thought to control KNDy neurons in a see-saw method to generate the pulsatile activity of KNDy neurons in the ARC. But this hypothesis has not been proved in solid proof. In my present study, lesion of *Oprk1*-expressing cells in the ARC caused an increase of LH frequency but not the disappearance of LH valley, in other words, continuous LH secretion. Similarly, previous studies showed that neither knockout of Dyn or KOR resulted in infertility in the mice [96–98]. The fact that these knockouts were achieved in mice made it difficult to see what exactly the LH pulse looks like after losing the “stop” signal. But LH level in these KO mice after OVX was lower than that in wildtype mice [47], so it is hard to consider this result as a direct consequence of losing the “stop” signal. It is reasonable to hypothesis that besides Dyn-KOR signaling, there is some compensate pathway to keep the LH from being too high.

Intracellular response of *Oprk1*-expressing cells to Dyn stimulation

Results in Chapter 4 showed that intracerebroventricular (i.c.v) injection of nalfurafine, a KOR agonist, significantly increased the number of the cells that co-express c-Fos and *Oprk1*. This results demonstrated that *Oprk1*-expressing cells were activated by the administration of nalfurafine.

Although Dyn was previously thought to inactivate cells that contain KOR, other studies demonstrated the possibility of alternative pathways downstream to KOR that increase cell activity (Fig. 5-2). KOR was recognized to activate the most kinds of mitogen-activated protein kinases (MAPKs) pathways [82–85]. Two members of the MAPK, extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (p38 MAPK) have been proved to affect c-Fos directly. ERK is able to increase the expression of c-Fos gene [86] and phosphorylate c-Fos to increase its stabilization and activation [87]. In addition, p38 MAPK was also shown to be able to increase phosphorylation of c-Fos [88]. Although no researches discussed the signal pathway from KOR all the way down to c-Fos and cellular activity, these previous studies on the MAPK pathways corresponds to my current result (Fig. 4-3) and explains the increase of co-expression of *Oprk1* and c-Fos.

G protein-coupled inwardly-rectifying potassium channel (GIRK) has been proved to be another effector of KOR [78,79]. This channel was previously thought to be activated by Dyn and results in suppression of cell activity, in the same manner as another famous GIRK, the cardiac channel that is activated by acetylcholine through M2 muscarinic receptors [81]. Recent studies demonstrated that MAPK pathway provides an alternative reaction to GIRK. Studies in spinal cord [92,93] and neurons

in dorsal raphe nucleus [94] showed that activation of p38 MAPK pathway by KOR stimulates GIRK phosphorylation and results in inhibited conduction of GIRK (Fig.5-2). Since expression of c-Fos is generally used as an indirect marker of neuronal activity because c-Fos is often expressed when neurons are stimulated [75,76], the present results demonstrated that *Oprk1*-expressing cells in the ARC were activated when combined with the ligand of KOR.

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.

Figures

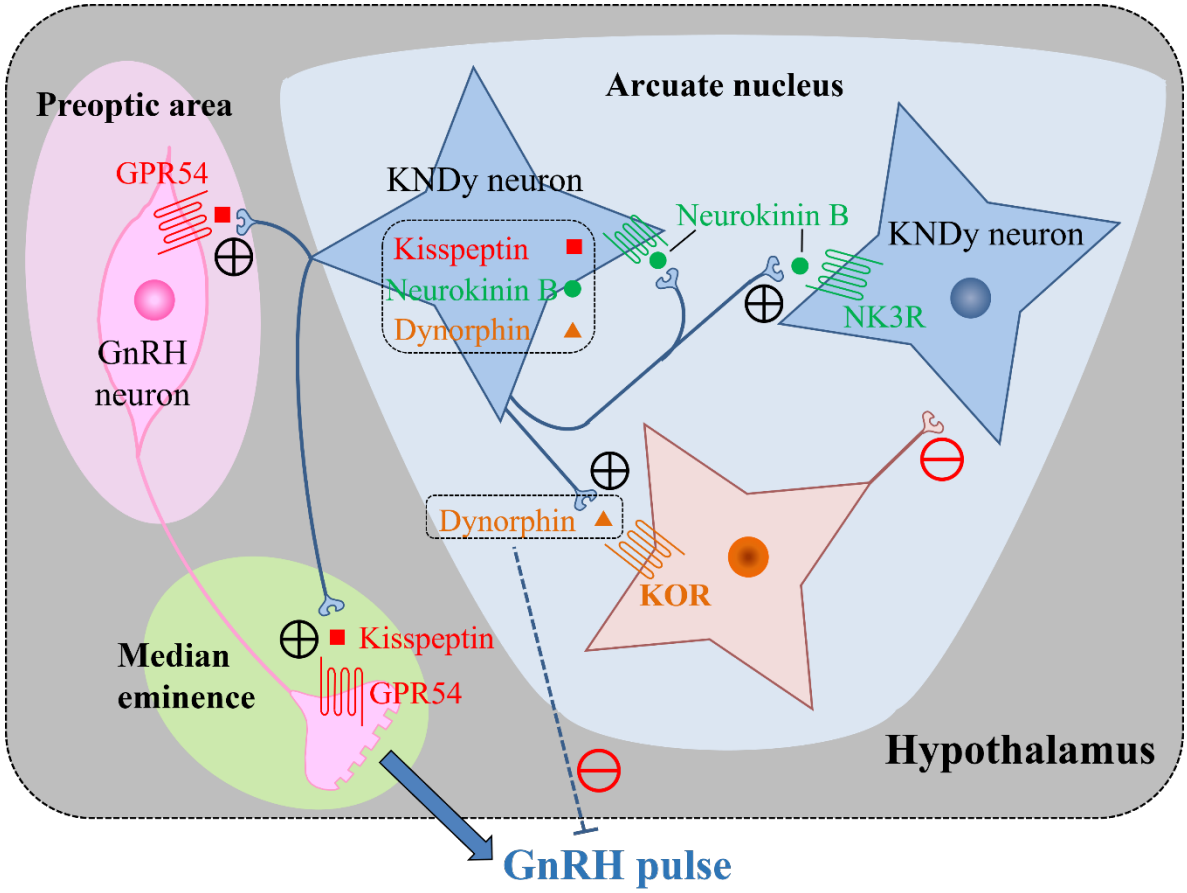

Figure 5-1. Schematic illustration of neural circuit involves *Oprk1*-expressing cells (orange colored) and KNDy neurons in the ARC (blue colored). According to this model, KNDy neurons in the ARC form a neural circuit by their collaterals and dendrites. NKB/NK3 signaling plays the role of accelerator in autocrine or paracrine manner, whereas *Oprk1* is expressed in another kind of cells instead of KNDy neurons. *Oprk1*-expressing cells are activated by Dyn secreted from KNDy neurons, and suppress activation of KNDy neurons in return. Dotted line with a short bar at the end represent indirect suppressive effect. KOR, kappa opioid receptor; KNDy neuron, kisspeptin/NKB/Dyn neuron.

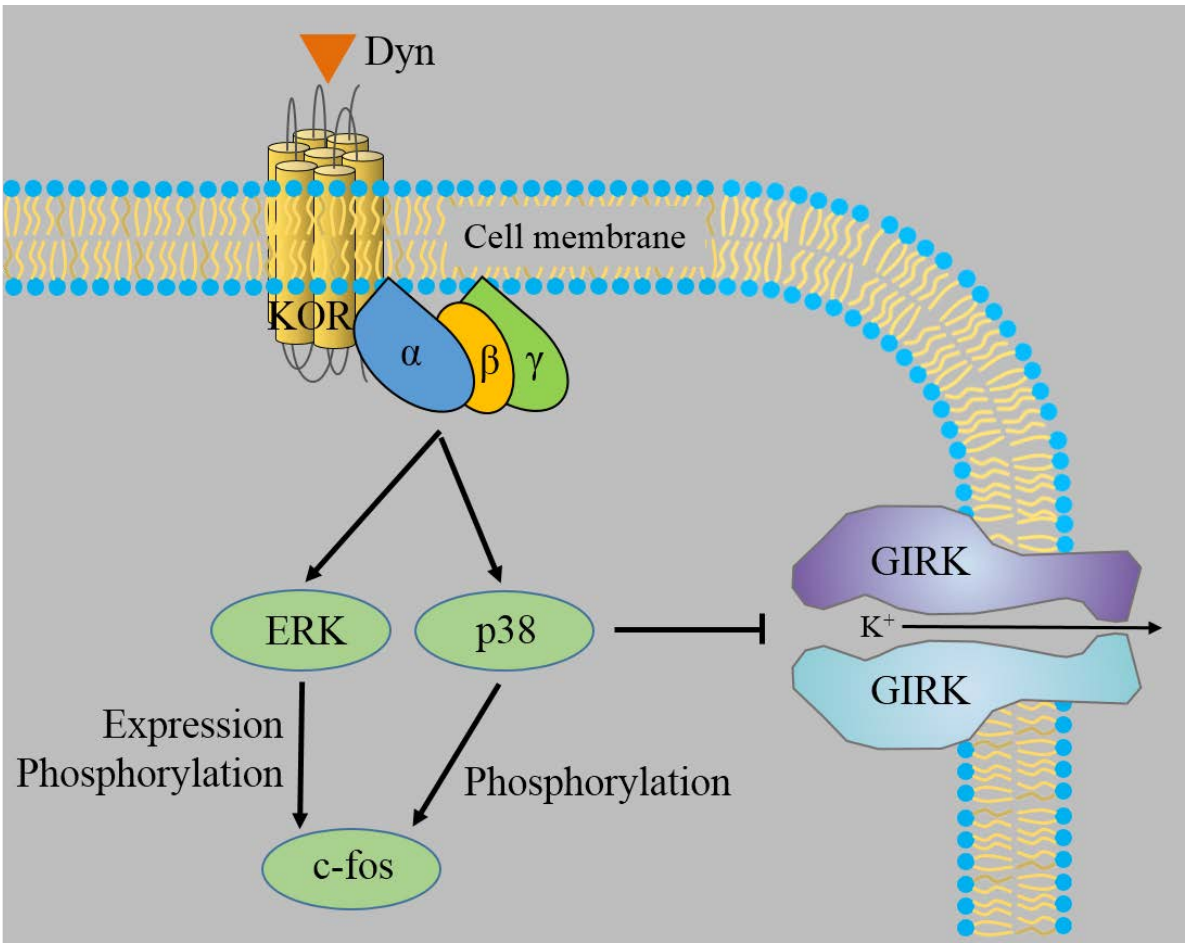

Figure 5-2. Schematic illustration of intracellular response of *Oprk1*-expressing cells to Dyn stimulation. According to this model, KOR activate ERK and p38 MAPK through G protein (shown as α, β and γ subunits in blue, orange and green, respectively) after bond to Dyn. ERK then increase the expression of *c-fos* and c-Fos phosphorylation, while p38 promote phosphorylation. p38 can also suppress the conduction of GIRK to stop the potassium ion from going out of the cytoplasm. ERK, extracellular signal-regulated kinase; Dyn, dynorphin; GIRK, G protein-coupled inwardly-rectifying potassium channels; KOR, kappa opioid receptor.

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