学位論文 （要約）

# Neuroendocrinological studies on the central regulatory mechanisms of teleost reproduction with special reference to hypothalamic GnRH neurons

# （視床下部 GnRH ニューロンを中心とした真骨魚類生殖中枢制御機構に関する神経内分泌学的研究）

平成 25 年 12 月博士（理学）申請

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# Abstract

Animals receive various information from their environment and adapt to it by changing their internal homeostatic regulations. Among them, adaptive regulation of breeding conditions depending on both internal and external environments such as seasonality and nutrition, is one of the most important examples of such adaptations. The hypothalamic-pituitary-gonadal (HPG) axis is the central regulator of reproduction in almost all vertebrates, and GnRH1 neurons in the hypothalamus have been suggested to play a central role in the HPG axis regulation. In order to understand the central regulatory mechanisms of reproduction, the basic properties of GnRH1 neurons themselves have been studied mainly in rodents. However, the analysis of the neural circuitry for the HPG axis regulation appears to be incomplete mainly because of the technical limitation in mammalian studies. In the present thesis, to overcome such problems in the previous studies and to reveal the general principles underlying the HPG axis regulation mechanisms of vertebrates in common, I analyzed the HPG axis regulation mechanisms focusing on the hypophysiotropic GnRH1 neurons and pituitary gonadotropes by taking advantage of teleosts for the neuroendocrinological studies.

In Chapter 1, I analyzed the general properties of GnRH1 neurons and gonadotropin secretion during the ovulatory cycle using medaka as a model. First I recorded the electrical activities of GnRH1 neurons in the ventral preoptic area (vPOA), which directly project to the pituitary, and found that the spontaneous firing activities of vPOA GnRH1 neurons showed time-of-day-dependent changes; the firing rate in the afternoon was higher than that in the morning. Also, I examined the daily changes in the pituitary gonadotropin transcription level and

found that the expression levels of *lhb* and *fshb* mRNA varied according to the time of day, peaking during night. I also demonstrated that GnRH increases *lhb* mRNA transcription several hours after GnRH stimulation in an isolated whole pituitary culture, which explains the time gap between the peaks of GnRH1 release and *lhb* mRNA transcription. From these results, I proposed a working hypothesis concerning the temporal regulation of the ovulatory cycle in the brain and pituitary of female medaka.

After analyzing the basic properties of GnRH1 neurons and gonadotropin gene transcription during the ovulatory cycle in medaka, I analyzed the effects of GnRH in the pituitary concerning hormone secretion in Chapter 2. In order to analyze the effect of GnRH on LH and FSH cells separately, I first generated transgenic medaka lines that express a fluorescent $Ca^{2+}$ indicator protein, inverse-pericam, specifically in the LH cells or FSH cells. By using these transgenic lines, I performed cell-type specific $Ca^{2+}$ imaging and demonstrated that LH and FSH cells show different $Ca^{2+}$ dynamics in response to GnRH. The results suggested that LH and FSH release are regulated by GnRH in different manners.

Finally, in Chapter 3, I searched for the regulator of GnRH1 neurons. As the candidate transmitters conveying neuronal inputs to the GnRH1 neurons, I focused on a neuropeptide kisspeptin and a monoaminergic neurotransmitter dopamine. Kisspeptin is known as one of the most potent activators of HPG axis in mammals, but it was shown that, in neither medaka nor goldfish, the kisspeptin administration *in vivo* induced an increase in the plasma LH concentration nor ovulation, and the kisspeptin administration *in vitro* did not change the GnRH1 neuronal activities at all. These results suggest that kisspeptin is dispensable for stimulation of the teleost GnRH1 neurons, at least in the same manner as in

mammals. On the other hand, I demonstrated that dopamine administration significantly inhibits the activities of GnRH1 neurons. The dopaminergic fibers projected to the cell bodies of the vPOA GnRH1 neurons and to the pituitary by intertwining with the GnRH1 fibers in the pituitary. Taken together, dopamine neurons possibly inhibit the GnRH1 neurons as well as the gonadotropes, suggesting that the dopaminergic inhibition of gonadotropin secretion is conserved among mammalian and teleost species.

In my thesis, I revealed several novel aspects of the HPG axis regulation mechanisms with special focus on the hypophysiotropic GnRH1 neurons and the pituitary, by taking advantage of medaka as a model system for the study of such mechanisms in vertebrates.

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# Abbreviations

| | |
|---|---|
| 2APB | 2-aminoethoxydiphenyl borate |
| ACSF | artificial cerebrospinal fluid |
| BAC | bacterial artificial chromosome |
| BCIP | 5-bromo-4-chloro-3-indoyl-phosphate |
| DIG | digoxigenin |
| Dl | area dorsalis telencephali pars lateralis |
| Dm | area dorsalis telencephali pars medialis |
| Dp | area dorsalis telencephali pars posterior |
| E2 | 17β-estradiol |
| EGFP | enhanced green fluorescent protein |
| FSH | follicle stimulating hormone |
| GFP | green fluorescent protein |
| GnRH | gonadotropin-releasing hormone |
| gpa | glycoprotein α |
| GSI | gonadosomatic index |
| GTH | gonadotropin |
| HPG axis | hypothalamic-pituitary-gonadal axis |
| hrGFP | humanized renilla green fluorescent protein |
| i.c.v. | intracerebroventricular administration |
| i.p. | intraperitoneal administration |
| IHC | immunohistochemistry |
| IP | inverse-pericam |
| $IP_3$ | inositol 1,4,5-trisphosphate |
| ir | immunoreactive |
| ISH | *in situ* hybridization |
| LH | luteinizing hormone |
| mdGnRH | medaka GnRH |
| NBT | 4-nitroblue tetrazolium chloride |
| OT | tectum opticum |
| OVX | ovariectomy |

| | |
|---|---|
| PBST | phosphate buffered saline with tween 20 |
| PKA | protein kinase A |
| PKC | protein kinase C |
| POA | preoptic area |
| POm | nucleus preopticus pars magnocellularis |
| POp | nucleus preopticus pars parvocellularis |
| PPD | proximal pars distalis |
| RGS | regulators of G protein signaling |
| TH | tyrosine hydroxylase |
| Vp | area ventralis telencephali pars posterior |
| vPOA | ventrolateral POA |

# General Introduction

Animals receive diverse information from the external environments and change their internal conditions for the adaptation to the environments, which enable them to respond appropriately to the external cues. Among such regulations, the control of reproduction is one of the most important regulations for the animals. Especially, the seasonally breeding animals show dynamic changes in the serum hormone concentrations and the gonadal size according to the seasonal cues such as day length, temperature, and nutrition.

The neuroendocrine control mechanisms of reproduction have been addressed intensely for a long time, and the hypothalamic-pituitary-gonadal axis (HPG axis) is believed as the "central dogma" in the central regulation of reproduction in almost all vertebrate species. Since the identification of gonadotropin releasing hormone (GnRH) by Schally and Guillemin in the 1970's (1-3), GnRH neurons have been considered as the final common pathway in hypothalamus for the control of reproduction. The function of hypophysiotropic GnRH neurons (called as GnRH1 neurons in most animals) is well-conserved among vertebrates. GnRH is a neuropeptide produced by GnRH1 neurons, which are located in the basal hypothalamic and/or preoptic area (POA) (4). The GnRH1 neurons stimulate gonadotropin secretion from the pituitary. In mammals, the GnRH1 neurons are located in the hypothalamic region and project their axons to the median eminence, and GnRH1 peptide is conveyed to the pituitary via the portal vessel (4). In teleost fish, the hypothalamic GnRH1 neurons directly innervate the pituitary (5-7) and stimulate the gonadotropin secretion from the gonadotropes. Two types of gonadotropins, luteinizing hormones (LH) and follicle stimulating hormone (FSH) are released from the pituitary and stimulate ovulation or follicle maturation respectively in females (illustrated in Fig. 0).

The earlier studies on the GnRH1 system were the histological analysis and the measurement of the amount of released hormones in the serum, gonadotropins, and GnRH, after intracerebroventricular applications or treatment of the hypothalamic explants or cultures with candidate regulatory factors of the HPG axis. Because of the technical limitations in those days, further analysis on the intrinsic electrical properties of the GnRH1 neurons and their neural networks has not been studied at the cellular level. However, physiological studies of GnRH1 neurons were greatly facilitated after the generation of transgenic mice and rats expressing green fluorescent protein (GFP) in GnRH1 neurons around the beginning of the 21st century (8-11). After the generation of transgenic animals, considerable numbers of electrophysiological analyses of GnRH1 neurons have been performed especially in mice and rats. The GFP transgenic labeling technique of GnRH1 neurons brought about a breakthrough in the cellular analysis of the features of GnRH1 neurons themselves under specific physiological conditions. However, the elucidation of the neural circuit for the HPG axis regulation is still difficult. This is partly because the brains of rodents are too large to perform experiments while keeping the neural circuitries intact, which, therefore, necessitates preparation of brain slices; the neural circuits are inevitably disrupted during the preparation of thin brain slices. Recently, it has been gradually clarified that a knowledge obtained in rodents is not necessarily consistent with those in other species, and there are some species variations in the HPG axis regulatory mechanism even in mammals. This is exactly the reason we should examine and compare results from various species, instead of using just a few specific animals such as rodents. Unfortunately, the cellular level studies on the HPG axis regulation in the non-mammalian vertebrates has lagged behind those in the mammalian species because of the lack of appropriate model

animal species, and only one report has been published concerning the non-mammalian GnRH1 neuronal activities in a cichlid fish (12) before I started the present work.

To overcome these previously described problems and to study the general principles underlying the HPG axis regulation mechanisms of vertebrates in general, I started to study the regulation mechanisms of gonadotropes by the GnRH1 neurons, by making the most of the following advantages of teleosts for the study of reproductive endocrinology. First, the gonadotropins, LH and FSH, are secreted from distinct populations of gonadotropes, LH and FSH cells, that express those hormones separately (13), whereas LH and FSH are secreted from a single type of pituitary gonadotropes in mammals, which makes the study of control mechanisms of different gonadotropins difficult. Second, the adenohypophysis is directly innervated by the neurosecretory fibers in teleosts, and is not intercalated by portal vessels as in mammals (14). These features are helpful for the analysis of hypophysiotropic neural control of gonadotrophins at the single cell level. Among the teleost species, medaka has the following advantages for neuroendocrinological analyses of reproduction. First, various molecular genetic tools such as transgenic technologies are available. Second, their small and transparent brains enable us to use a whole-brain in vitro preparation, in which intact neural circuits are maintained. Third, their 1-day ovulatory cycle facilitates the analysis of the regulatory mechanisms of GnRH1 neurons and gonadotropins in rather natural and physiological conditions throughout the ovulatory cycle. Therefore, I started the present thesis work by using medaka as a teleost model.

To begin with, I started my study by examining the characteristics of GnRH1 neurons and gonadotropin secretion during the ovulatory cycle of medaka, because

there had been no report on the neural activity of GnRH1 neurons and gonadotropin secretion during natural ovulatory cycles in any vertebrate species. By retrograde labeling of the neurons projecting to the pituitary, I found that GnRH1 neurons in the ventral preoptic area (vPOA) directly project to the pituitary. I therefore recorded the neural activities from these GnRH1 neurons using transgenic medaka, in which GnRH1 neurons are labeled by GFP. Taking advantage of 1-day ovulatory cycle of medaka, I recorded neural activities of GnRH1 neurons during ovulatory cycle and found that the spontaneous firing activity of GnRH1 neurons showed time-of-day-dependent changes: the firing activity in the afternoon was higher than in the morning. Also, I examined the daily changes in the pituitary gonadotropin transcription level. The expression levels of *lhb* and *fshb* mRNA also showed changes related to the time of day, peaking during the lights-off period. Next, I analyzed effects of GnRH on the pituitary gonadotropes. I demonstrated that GnRH increases *lhb* mRNA transcription several hours after the stimulation in isolated pituitary preparations, which was very slow compared to the well-known immediate LH releasing effect of GnRH. From these results, I proposed a working hypothesis concerning the temporal regulation of the ovulatory cycle in the brain and pituitary of female medaka (Chapter 1).

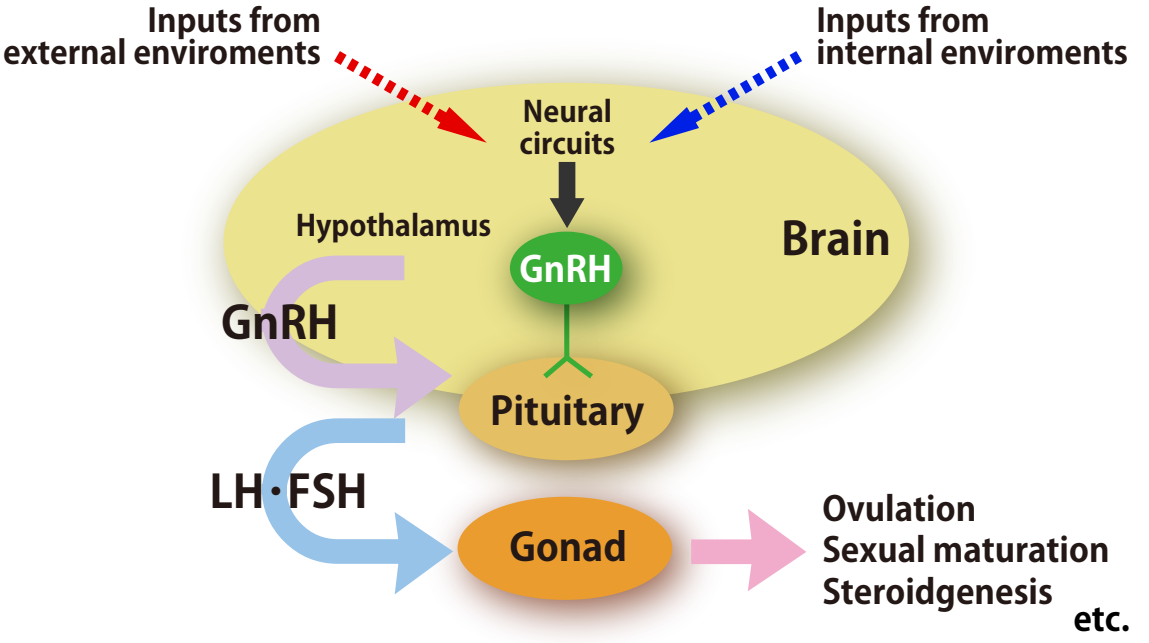
In Chapter 2, I analyzed the control mechanisms of GnRH on the release of LH and FSH, respectively. Two types of gonadotropins, LH and FSH are important pituitary hormones for sexual maturation and reproduction, and both of them are known to be centrally regulated by GnRH. However, the mechanisms of differential regulation by GnRH of the release of two types of gonadotropins with different secretory profiles are still unknown. Taking advantages of teleost pituitary, where LH and FSH are secreted from separate cellular populations unlike in mammals, I

generated transgenic medaka lines that express $Ca^{2+}$ indicator proteins, inverse-pericam, specifically in the LH or FSH cells. I performed cell-type specific $Ca^{2+}$ imaging of LH and FSH cells, respectively. Using the whole brain-pituitary preparations of these transgenic fish in which all neural circuits and GnRH neuronal projection to the pituitary are kept intact, I found that LH and FSH cells showed different $Ca^{2+}$ responses to GnRH. I also succeeded in detecting the effect of endogenous GnRH peptide on LH cells, which was released by electrical stimulation of the axons of GnRH1 neurons. The results suggest the differential regulatory mechanisms for LH and FSH release by GnRH and that neural control of LH release by GnRH1 neurons are functionally important *in vivo* in animals (Chapter 2).

GnRH neurons may alter their firing activity according to the environmental cues or the animals' conditions to alter their reproductive states. However, only a few neuronal inputs have been identified as regulators of GnRH1 neurons. To understand how animals change their reproductive state via GnRH1 neurons, I searched for the candidate neural inputs to the GnRH1 neurons. First, I focused on the neuropeptide kisspeptin, which is recently known as a potent activator of GnRH1 neuronal activity in mammals. However, *in vivo* intraperitoneal or intracerebroventricular administration of kisspeptin failed to induce plasma LH elevation or ovulation in sexually mature female goldfish. Moreover, electrophysiological experiments showed that Kiss1 did not change the firing frequency of GnRH1 neurons in medaka. Thus, it was strongly suggested that in some teleosts kisspeptins are dispensable for LH release at least in the same manner as reported in mammals. Next, I focused on a monoamine neurotransmitter dopamine, which had been suggested to have inhibitory effects on the HPG axis of

some vertebrates. I found close appositions between the dopaminergic fibers and the GnRH1 cell bodies in vPOA as well as the characteristic association of projecting dopaminergic and GnRH fibers in the pituitary. I also demonstrated electrophysiologically that dopamine inhibits the vPOA GnRH neuronal activity. In addition, LH and FSH cells in the pituitary received dopaminergic projections (Chapter 3). These results suggest that dopamine neurons possibly inhibit the HPG axis at two levels: at the level of GnRH1 neuron cell bodies in the POA, and at the level of the gonadotropes in the pituitary.

Figure 0


Inputs from
external enviroments
Inputs from
internal enviroments
Neural
circuits
Hypothalamus
GnRH
Brain
GnRH
Pituitary
LH·FSH
Gonad
Ovulation
Sexual maturation
Steroidgenesis
etc.

# Chapter 1

## Time-of-Day-Dependent Changes in GnRH1 Neuronal Activities and Gonadotropin mRNA Expression in Medaka: Estrous Cyclicity

## Abstract

Gonadotropin-releasing hormone (GnRH) neurons in the preoptic area and hypothalamus control the secretion of GnRH and form the final common pathway for hypothalamic–pituitary–gonadal (HPG) axis regulation in vertebrates. Temporal regulation of reproduction by coordinating endogenous physiological conditions and behaviors is important for successful reproduction. Here, I examined the temporal regulation of reproduction by measuring time-of-day dependent changes in the electrical activity of GnRH1 neurons and in the levels of expression of pituitary gonadotropin mRNA using a daily spawning teleost, medaka (*Oryzias latipes*). First, I performed on-cell patch clamp recordings from GnRH1 neurons that directly project to the pituitary, using *gnrh1*-GFP transgenic medaka. The spontaneous firing activity of GnRH1 neurons showed time-of-day dependent changes: overall, the firing activity in the afternoon was higher than in the morning. Next, I examined the daily changes in the pituitary gonadotropin transcription level. The expression levels of *lhb* and *fshb* mRNA also showed changes related to time of day, peaking during the lights-off period. Finally, I analyzed effects of GnRH on the pituitary. I demonstrated that incubation of isolated pituitary with GnRH increases *lhb* mRNA transcription several hours after GnRH stimulation, unlike the well-known immediate LH releasing effect of GnRH. From these results, I propose a working hypothesis concerning the temporal regulation of the ovulatory cycle in the brain and pituitary of female medaka.

## Introduction

Gonadotropin-releasing hormone (GnRH) neurons of the preoptic area and hypothalamus are responsible for the secretion of GnRH and form the final common pathway in the central regulation of reproductive functions. A rapid and large increase in GnRH levels, the GnRH surge, is a trigger for a surge in pituitary luteinizing hormone (LH) and a prerequisite for ovulation. This preovulatory LH surge has been demonstrated in many vertebrate species (15-21).

Temporal regulation is important for successful reproduction, and numerous physiological and behavioral processes that depend on neuronal and hormonal activities must be coordinated to achieve that goal. For example, ovulation, sexual behavior, fertilization, and maintenance of pregnancy require specific temporal patterns of hormone secretion in spontaneous ovulators such as rats, hamsters, and mice (22-24). A good example of temporal regulation of reproduction is the regulation of the daily LH surge, which has been observed in many vertebrates. The LH surge in most women begins early in the morning (25). Similarly, in nocturnal rodents, the LH surge begins immediately preceding their active period, around the time of lights off (26). Comparable phenomena are known to occur in non-mammals; for example, goldfish show an LH surge at dawn on the day of spawning (21). The mechanisms that control the occurrence of an LH surge at particular time of day are thus suggested to be conserved throughout the evolutionary lineage of vertebrates, but are not well understood.

In the present study, I examined the temporal regulation of reproduction by measuring time-of-day dependent changes in the electrical activity of GnRH1 neurons and in the levels of expression of pituitary gonadotropin mRNA using a

daily spawning teleost, medaka. Medaka is a diurnal teleost that spawns every morning under breeding condition and presents several advantages for the study of temporal regulation mechanisms of reproduction. First, their one-day ovulatory cycle facilitates the analysis of the regulatory mechanisms of GnRH1 neurons and gonadotropins in rather natural and physiological conditions throughout the ovulatory cycle. In addition, their small and transparent brains enable us to use a whole-brain *in vitro* preparation, in which intact neural circuits are maintained for electrical recording and imaging.

I found time-of-day dependent changes in the firing activities of GnRH1 neurons and transcription of pituitary gonadotropin mRNA. The increased activity of GnRH1 neurons in the afternoon is suggested to rapidly induce the LH surge in the same day and also to induce transcription of *lhb* mRNA after a delay to promote synthesis of LH that becomes the source for the LH surge in the next ovulatory cycle. This is the first report to strongly suggest a relationship between GnRH1 neuronal activity and LH secretion by examining their time-of-day dependent changes throughout the ovulatory cycle.

## Materials and Methods

### *Animals*

Male and female wild-type d-rR medaka (*Oryzias latipes*), *gnrh1:hrGFP* transgenic medaka (27), and *gnrh1*:EGFP transgenic medaka were maintained in pairs under a 14-h light and 10-h dark photoperiod (light on at 8:00 and light off at 22:00) with a water temperature of 27 °C. Female subjects were sexually mature (body weight: 0.22 ~ 0.26 g) and spawned for at least three consecutive days [gonadosomatic index

(GSI): 8.0 ~ 14.0]. Fish were fed two to three times daily with live brine shrimp and flake food. All animals were maintained and used in accordance with guidelines established by the University of Tokyo for the use and care of experimental animals.

### *Histology*

To identify GnRH1 neurons in the POA, I performed *in situ* hybridization using a DIG labeled RNA probe that specifically labels medaka *gnrh1* mRNA following a standard protocol (28) with some minor modifications. After DIG-3 treatment, I added an alkaline phosphatase substrate, NBT/BCIP (337 mg/ml NBT and 175 mg/ml BCIP) in DIG-3. I then took photographs at 10 min, 30 min and 240 min after application of NBT/BCIP, while the reaction continued. Innervation of the pituitary by GnRH1 neurons was examined using a dual fluorescence method by retrograde labeling of biocytin and GnRH immunohistochemistry (or *in situ* hybridization) as described previously (29). Briefly, I anesthetized fish and dissected out the whole brain and pituitary of male and female d-rR medaka and then inserted a small crystal of biocytin (Sigma, St. Louis, MO) into the pituitary. After incubation of the brain in fish artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 134, KCl 2.9, $CaCl_2$ 2.1, $MgCl_2$ 1.2, HEPES 10, and glucose 15 (adjusted to pH 7.4 with NaOH) for 30 - 60 min, brains were fixed with 4 % paraformaldehyde in PBS. The fixed brain was sectioned frontally at 30 μm using a cryostat (CM 3050S, Leica microsystems, Wetzlar, Germany) and mounted onto MAS-GP typeA coated glass slides (Matsunami, Osaka, Japan). Then, the retrogradely labeled biocytin signals were visualized with AlexaFluor 488 conjugated streptavidin (1:500; Invitrogen, Carlsbad, CA) by reaction with ABC *Elite* kit (Vector, Burlingame, CA). GnRH immunoreactivity was visualized by anti-medaka (md)GnRH antiserum (1:10,000)

and AlexaFluor555 conjugated anti-rabbit IgG (1:800; Invitrogen). The fluorescent signals were observed using a LSM-710 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany). Anti-mdGnRH (GnRH1) antiserum was produced as follows; mdGnRH peptide (pE-H-W-S-F-G-L-S-P-G-$NH_2$) was synthesized chemically by Sawady Technology (Tokyo, Japan). An antiserum against mdGnRH (lot no. 2) was raised against the full-length mdGnRH decapeptide by immunization of a rabbit (Gift from Dr. Kataaki Okubo, The University of Tokyo, (7)). The specificity of the antiserum was assessed by a dot blot analysis. Briefly, synthetic mdGnRH (GnRH1), chicken-type GnRH (GnRH2), and salmon-type GnRH (GnRH3) peptides were serially diluted 1:5 from a starting concentration of 500 ng/μl down to a concentration of 160 pg/μl, and 1 ml of these peptide solutions were spotted on nylon membranes (Hybond-$N^+$; Amersham Pharmacia Biotech, Amersham, UK). The subsequent immunoreaction with the antiserum against mdGnRH was performed in the same manner as for the tissue samples, as described below. This dot blot test demonstrated that the mdGnRH antiserum cross-reacted with chicken-type GnRH and salmon-type GnRH by less than 0.8% (data not shown). I also used fluorescent *in situ* hybridization with an HNPP (2-hydroxy-3-naphthoic acid-2′- phenylanilide phosphate) fluorescence detection kit (Roche) to label GnRH1 neurons. The results of *in situ* hybridization were consistent with that of immunohistochemistry. I used wild type d-rR medaka for all histological experiments.

### *Electrophysiology*

For the electrophysiological analysis, I used both a *gnrh1*:hrGFP transgenic line (27) and a *gnrh1*:EGFP transgenic line. Because the two transgenic lines showed

similar results, I combined all data from the two lines. Fish were anesthetized by immersion in tricaine methanesulfonate (MS-222; dissolved 0.02% in tap water) and then decapitated. *In vitro* whole brain preparation for the patch-clamp recordings from genetically GFP-labeled GnRH1 neurons was carried out as follows. First, the brain and attached pituitary were dissected out and placed in a hand-made chamber filled with ACSF. Then, the optic nerve was removed from the brain to make it easier for the patch pipettes to access the brain, and the ependymal layer of preoptic area was carefully peeled off with fine forceps. Under an upright fluorescent microscope with infrared differential interference contrast optics (Eclipse E600FN, Nikon, Tokyo, Japan), the exposed GnRH1-GFP neurons were easily identified visually from the ventral side of the brain. The patch pipette was carefully directed toward the GnRH1-GFP neurons with the aid of an MP-225 micromanipulator (Sutter Instruments, Novato, CA) by comparing the images on the infrared differential interference contrast monitor and the GFP fluorescence through the ocular lens. Patch pipettes of borosilicate glass capillaries with 1.5-mm outer diameter (GD-1.5; Narishige, Tokyo, Japan) were pulled using a Flaming-Brown micropipette puller (P-97; Sutter Instruments, Novato, CA). The tip resistance of patch pipettes in ACSF was about 8~15 MΩ, and seal resistances for on-cell recordings were about 15 to 60 MΩ. ACSF was also used as the pipette solution for loose-patch recordings. Targeted single-unit extracellular loose-patch recordings were performed with patch-clamp amplifiers (CEZ-2400; Nihon Koden, Tokyo, Japan and and BVC-700A; Dagan, Minneapolis, MN) and I analyzed spontaneous firing activity of single GnRH1-GFP neurons extracellularly by continuously recording changes in currents that are associated with action potential generation (action currents). The action currents were filtered at 1 kHz with a

low-pass four-pole Bessel filter and digitized at 10kHz using a Digidata 1320A interface and pClamp8 software (Molecular Devices, Sunnyvale, CA). All recordings were initiated within two hours after dissecting out the whole brain with pituitary. Electrical recordings were performed every four hours (n=10~13 for each time point).

*Quantification of time-of-day dependent changes in pituitary gonadotropin mRNA*

Female medaka were sacrificed under MS-222 anesthesia, and the pituitaries were collected for real-time PCR analysis. Pituitaries were collected six times a day, every four hours (1:00, 5:00, 9:00, 13:00, 17:00, and 21:00), from 14 or 16 fish per each sampling. Total RNA was extracted from the pituitaries using the FastPure RNA kit (Takara, Shiga, Japan) according to the manufacture's protocol. Two pituitaries were homogenized in a tube by vortexing with the FastPure kit lysis buffer for more than one minute (n=7 or 8 for each time point). Genomic DNA was removed by DNaseI (Ambion, Applied Biosystems, Foster City, CA) treatment on a column membrane. Total RNA was reverse-transcribed with a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time PCR was performed as previously described (30). The PCR product was verified by the melting curve analysis. The data were normalized to a housekeeping gene, *b-actin*, and average expression levels were calculated. Primer pairs used for real-time PCR are listed in Table 1-1 and were designed as follows: (i) one side of the primer pairs was designed in the exon-exon boundary, and the other one was designed inside of the exon to detect mature mRNA and (ii) the both primer pairs were designed in the same intron to detect the mRNA precursor.

### *GnRH effects on lhb mRNA and lhb primary transcript transcription in the isolated pituitary*

I collected the pituitaries at around 10:00 and immediately began pre-incubation in normal medium (NM; Leibovitz's L-15 medium (pH 7.4; cat. no. L5520; Sigma) supplemented with L-glutamine (2 mM), D-glucose (10 mM), penicillin-streptomycin (100 U of penicillin and 0.1 mg streptomycin/ml; Life Technologies), and 5% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) at 25 °C for two hours. 10 nM 17β-estradiol (E2) was added for control group (C group), and 10 nM E2 and 100 nM mdGnRH for GnRH administration group (G group; n=5~8, two pituitaries for each sample). Given the data available for goldfish (21), I expect that 10 nM E2 would be a reasonable physiological level for a female teleost. I determined the concentration of synthetic mdGnRH peptide by reference to (31): application of 100 nM GnRH induces rapid intracellular calcium rise in dissociated goldfish gonadotropes. After two-hour preincubation with or without mdGnRH, each sample was washed with NM and then incubated in NM for 0, 4, 8, 12, or 16 hours. The experimental scheme is shown in Fig. 1-4A. After incubation, the pituitaries were homogenized, and *lhb* mRNA was quantified in the same manner as were time-of-day dependent changes in pituitary gonadotropin mRNA. Because *lhb* mRNA level was greatly increased by co-treatment of GnRH with estrogen (32-35), I added estrogen to NM when isolated pituitaries were pretreated with GnRH. I also performed preliminary tests to show that estrogen treatment alone did not change *lhb* mRNA expressions (data not shown).

### *Data analysis*

All values are shown as mean ± SEM. For electrophysiological experiments, the action currents were recorded between 5 and 10 or 15 min after the start of

recordings and were counted using Clampfit10.2 (Molecular Devices). The mean firing rate (spikes/seconds) was determined by dividing the total number of action currents detected during the 5 or 10 min recording period by the total recording time (seconds) (Fig. 1-2E). Instantaneous frequency (the interval between events, converted to frequency in Hz) was also calculated from the data for the 5 or 10 min recording period (Fig. 1-2F). Quiescence was defined as a 1-min bin containing one event or less. The percentage of bins that were quiescent (Fig. 1-2G), and the maximum duration of quiescence (number of consecutive quiescent bins) during the recording period (5 min or 10 min) (Fig. 1-2H) were determined for each recording. When quiescence continued for the whole duration of recording (5 min), the maximum duration of quiescence was defined as 5 min or 10 min. Because female medaka have a one-day ovulatory cycle, I arbitrarily categorized the recorded data into six groups, each with four-hour period (Fig. 1-2: group a, 23:30~3:30; b, 3:30~7:30; c, 7:30~11:30; d, 11:30~15:30; e, 15:30~19:30; and f, 19:30~23:30; n=10, 10, 12, 11, 12, and 13, respectively). The mean value for each group was calculated and multiple comparison tests were performed using the Tukey-Kramer test (significance levels < 0.05). For the analysis of the time-of-day dependent changes in the pituitary mRNA levels, the expression level for each time period was normalized by the average mRNA expression level at 5:00. Outliers for each time point and gene were excluded using the Smirnov-Grubbs test (significance levels < 0.05). Outliers represented less than one data point in each sample group. Tukey-Kramer multiple comparison tests were performed for each time period and gene (significance levels < 0.05). For the GnRH-induced increase of *lhb* mRNA levels in pituitary primary culture, each data point was normalized by the averaged expression level measured just after the pre-incubation without mdGnRH and outliers were excluded using the

Smirnov-Grubbs test (significance levels < 0.05). Outliers represented less than one data point in each sample group. Expression levels were analyzed using a two-way factorial ANOVA and Dunnet's post hoc test (significance levels < 0.05).

## Results

### *Ventral population of GnRH1 neurons project to the pituitary*

The axons of GnRH1 neurons project directly to the pituitary in teleosts; this is quite different from mammalian GnRH1 neurons, the axons of which project to the median eminence and stimulate gonadotrophs indirectly via hypophyseal portal vessels (29). Two major populations of GnRH1 neurons are present in the telencephalon of medaka: one in the region spanning area ventralis pars dorsalis, supracommissuralis, and posterior (Vd/Vs/Vp) of telencephalon (referred to as the dorsal POA in (27)) (Fig. 1-1A), and the other in the region ranging from the lateral part of area ventralis pars ventralis of telencephalon (Vv) to the ventrolateral POA (ventral POA in (27)) (Fig. 1-1B). In the present paper, I refer to these populations as the dorsal and the ventral populations of GnRH1 neurons, respectively. A schematic illustration of these two populations is shown in Fig. 1-1C.

I examined the localization of the hypophysiotropic GnRH1 neurons using double fluorescence analysis, with retrograde labeling of neurons after biocytin injections to the pituitary and either GnRH immunohistochemistry or *in situ* hybridization for GnRH mRNA. I embedded the crystal of biocytin into various areas of the pituitary and labeled the pituitary-projecting neurons. The results clearly demonstrate colocalization of GnRH immunoreactivity and retrograde labeling in a part of the ventral population of GnRH1 neurons (Fig. 1-1B). On the other hand, I did not find

any colocalization with retrograde labeling in the dorsal population (Fig. 1-1A). This result in adult medaka was consistent with a previous report that GFP-positive axons from the POA project to the pituitary in GnRH1-GFP medaka embryos (27). Because of inevitable technical limitations of the retrograde tracing method, I cannot completely discount the possibility of a pituitary projection from the dorsal GnRH1 neurons; however, at most it would be a minor projection compared with the strong projection from the ventral population of GnRH1 neurons.

I also conducted a semi-quantitative comparison of levels of expression of GnRH mRNA in the dorsal and ventral populations of GnRH1 neurons by comparing the time to visualize digoxigenin precipitates during *in situ* hybridization in identical sections (see (36) for this method). I obtained similar results after repeating the same experiments several times, and a representative image is shown in Fig. 1-1D. Because the ventral GnRH1 neurons were visualized in less than 10 min, while it took more than 30 min to visualize the dorsal GnRH1 neurons (Fig. 1-1D), I suggest that the ventral GnRH1 neurons express much higher level of GnRH1 mRNA than the dorsal GnRH1 neurons.

### *GnRH1 neurons show time-of-day dependent changes in electrical activities*

Because the hypophysiotropic GnRH1 neurons project to the pituitary and release GnRH to stimulate LH release from pituitary gonadotropes, changes in GnRH neuronal activities are considered to be the immediate indications of GnRH and LH release. I focused my electrical recordings on the vPOA GnRH1 neurons, and will simply refer to these cells as “GnRH1 neurons” in this thesis. Using a targeted loose-patch single-unit recording technique, I analyzed spontaneous firing activity of single GnRH1-GFP neurons extracellularly by continuously recording

action currents for up to 120 min. Virtually all the recorded GnRH1-GFP neurons (Fig. 1-2A) showed irregular spontaneous activities (n = 66 neurons from 41 fish; Fig.1-2B). Two GnRH1 neurons were quiescent during the recording period.

Electrical recordings were performed every four hours, and I found that spontaneous activity changes with time of day. The LH surge in female medaka has been roughly estimated to start from eight to nine hours after the time of light-on (37), which corresponds to 16:00 to 17:00 in our lighting schedule. I divided a day into six four-hour periods and categorized the data recorded during each period. Fig.1-2C and D show representative traces of spontaneous firing activity of GnRH1-GFP neurons at the time preceding the putative LH surge (at 13:00, group d) and at the time just before the putative LH surge (at 16:00, group e), respectively. GnRH1 neuronal activity is clearly low at 13:00 and high at 16:00. The data for every four-hour period, showing the mean firing rate, median instantaneous frequency, and percentage and maximum duration of time in quiescence, are shown in Fig. 1-2E–H. The mean firing rate of GnRH1 neurons was highest at the time just before and during the putative LH surge (group e: 1.42±0.82 spikes/second, n = 12) and the lowest at the time immediately preceding it (group d: 0.24±0.08 spikes/second, n = 11; Fig. 1-2E). The median instantaneous frequency was also highest at the time just before and during the putative LH surge (group e: 1.39±0.51 spikes/second, n = 12) and the lowest at the time preceding it (group d: 0.41±0.14 spikes/second, n = 11; Fig. 1-2F). GnRH1 neurons recorded during 11:30~15:30 showed the highest percentage of quiescence, compared with other times of day (statistically significant differences were found between groups a and d, and groups b and d, *: $p<0.05$; Fig. 1-2G). In addition, the maximum duration of quiescence in the recording period during 11:30~15:30 was the longest, compared with those of

other time periods (statistically significant differences were found between groups a and d, groups b and d, groups c and d, and groups e and d, *: $p<0.05$; Fig. 1-2H). Two neurons were quiescent for the entire recording period: one out of twelve GnRH1 neurons recorded in group c, and one out of thirteen GnRH1 neurons recorded in group f. Taking all these results into consideration (Fig. 1-2E~H), electrical activity obviously changes with time of day, with a significant increase in GnRH neuronal activity in the afternoon which may produce an associated increase in release of GnRH to the pituitary (see Discussion).

### *Time-of-day dependent changes in gonadotropin mRNA/primary transcript expression levels in the pituitary*

Time-of-day dependent changes in levels of expression of *lhb* and *fshb* mRNAs and their primary transcripts in the breeding female pituitaries were analyzed. *Lhb* and *fshb* mRNAs and primary transcripts in pituitaries were measured six times a day at 4-h intervals (1:00, 5:00, 9:00, 13:00, 17:00, and 21:00; n=8, 7, 8, 7, 7 and 8, respectively). For quantification of gene expression at better time resolution, I designed the PCR primer pairs in the introns so that I could selectively quantify the amount of mRNA precursor before splicing, referred to as the primary transcript (38).

Both *lhb* mRNA and the primary transcript showed clear time-of-day dependent changes (Fig. 1-3A). In *lhb* mRNA, expression level was highest at 5:00 and showed significantly lower levels from 17:00 to 1:00. The change in *lhb* primary transcript was time-shifted forward, and the transcription level peaked from 1:00 to 5:00 and showed significantly lower levels from 13:00 to 21:00. *Fshb* mRNA is supposed to peak at 5:00, similar to *lhb* mRNA, although I did not find statistical significance. The *Fshb* primary transcript also showed time-of-day dependent changes, with

transcription level highest at 5:00 and significantly lower from 17:00 to 1:00 (Fig. 1-3B).

These results suggest that 1) transcriptional levels of *lhb* and *fshb* change with time of day, 2) because of their shorter half-life period *lhb* and *fshb* primary transcript levels show more significant time-of-day dependent changes than those of *lhb* and *fshb* mature mRNA, and 3) the peak time is similar for *lhb* and *fshb* transcription, highest during the light-off period and lowest during the latter half of light-on period, with slightly different dynamics in which the *lhb* primary transcript started to increase earlier than *fshb* primary transcript and was kept longer at higher transcription level. Thus, the *lhb* and *fshb* expression levels may be differentially regulated.

### *GnRH upregulates the expression of lhb mRNA on a slow time scale*

In many vertebrates, GnRH is known to trigger release of LH from the pituitary via activation of GnRH receptors in the LH producing cells. In medaka, I have reported that the mdGnRH peptide triggers an intracellular calcium ($[Ca^{2+}]_i$) rise in LH producing cells in the pituitary within several seconds after mdGnRH application, which should reflect the fast release of LH from the pituitary (39). A rapid $[Ca^{2+}]_i$ rise after GnRH application has also been reported in dissociated goldfish gonadotrope cells (31). Therefore, I examined the effects of mdGnRH peptide on the transcription of *lhb* mRNA in isolated pituitary preparations. The expression levels of *lhb* mRNA started to increase twelve hours after 2-hour pre-incubation with 100 nM GnRH and was significantly different 16 hours after preincubation ($p<0.001$), whereas they did not show any changes after preincubation without GnRH (Fig. 1-4B). The amount of *lhb* primary transcript

started to increase eight hours after the end of preincubation with GnRH and became significantly different at 16 hours ($p<0.01$; Fig. 1-4B). This result indicates that the facilitatory effect of mdGnRH peptide on *lhb* transcription in the pituitary proceeds on a slower time scale than the rapid LH release after GnRH application, which takes nearly ten hours or more.

## Discussion

In the present study, I examined the mechanisms of temporal regulation of reproduction by analyzing time-of-day dependent changes in the electrical activity of GnRH1 neurons and transcription of *lhb/fshb* mRNA, as well as GnRH-induced changes in *lhb* transcription, using the teleost medaka, which spawn on a daily basis.

### *vPOA GnRH1 neurons directly regulate the pituitary in medaka*

By retrograde labeling of neurons after biocytin injection to the pituitary, I demonstrated that vPOA GnRH1 neurons directly project to the pituitary in medaka. This is consistent with the fact that *gnrh1* in teleosts is homologous to *GnRH1* in mammals, which plays a role in hypophysiotropic regulation (40). In addition, I have shown that ventral GnRH1 neurons have higher levels of expression of *gnrh1* mRNA than do those of the dorsal population. These data led us to conclude that the ventral population of GnRH1 neurons in the POA (vPOA GnRH1 neurons) play a major role in hypophysiotropic functions and mainly contribute to the stimulation of gonadotropin release, and I therefore recorded their electrical activities.

*Firing activity of medaka GnRH1 neurons shows time-of-day dependent changes*

Using a model teleost, medaka, I recorded the activities of GnRH1 neurons at various time of the day and found changes in their activity levels corresponding to the reproductive cycle. Medaka is the only vertebrate species in which all three subtypes of GnRH neurons have been genetically labeled by GFP. In medaka, non-hypophysiotropic GnRH neurons, GnRH2 and GnRH3 neurons, show regular firing activities (41, 42), as has been reported for the dwarf gourami (43); (see also review by (44)). On the other hand, I report here that the GnRH1 neurons show irregular and episodic spontaneous firing activities, which is distinct from that of the extrahypothalamic GnRH2 and GnRH3 neurons in medaka (Fig. 1-2B). Similar irregular and episodic firing activities have been reported in the GnRH1 neurons of other vertebrates such as mice, rats, monkeys and cichlid fish (9, 11, 12, 45, 46).

The spontaneous firing activity of medaka GnRH1 neurons clearly showed time-of-day dependent changes in frequency; the overall tendency was low in the morning and high in the afternoon. Similar to the strong correlation between the multiple unit activities (MUA) and the pulsatile release of LH (47), the electrical activity of a single GnRH1 neuron is also expected to be correlated with GnRH release from the GnRH1 neuron. Concerning the dynamic changes in the spontaneous activity of GnRH1 neurons, there are some reports on the occurrence of time-of-day dependent changes in GnRH1 neuronal activity using the daily surge mice model (48). A series of electrophysiological studies by Christian and Moenter (49) demonstrated that spontaneous firing in GnRH1 neurons was low in the morning (AM) and high in the afternoon (PM) in ovariectomized and estrogen treated (OVX+E) mice, and that no such difference is apparent in OVX mice.

Although the intact female mice show an LH surge every four to five days, the OVX+E mice show LH surge every PM. The time-of-day dependent changes in the firing activity in mice were thought to reflect an increase in GnRH release, which in turn triggers an LH surge. Their studies suggest that some kind of circadian neural mechanisms may be important for the regulation of GnRH neuronal activities (50). I surmise that there are two possible factors concerning time-of-day dependent changes in GnRH1 neuronal activities in general: changes in synaptic inputs to the GnRH1 neurons and the changes in the intrinsic excitability of the GnRH1 neurons themselves. In mice, the existence of both mechanisms has been suggested, which may exert synergistic effects on the neuronal activities of GnRH1 neurons (49, 51). The frequency of excitatory fast synaptic transmission to the GnRH1 neurons, especially excitatory GABAergic transmission, was found to be high in the PM (at the start of lights-off period) and low in the AM (during lights-on period) in OVX + E mice (50). Also, high voltage activated $Ca^{2+}$ currents in the GnRH1 neurons varied with the time of day: currents in GnRH1 neurons recorded from OVX + E mice were lower than those from OVX mice in the AM but were higher in the PM (52). These results were consistent with an increase of GnRH neuronal activity preceding LH surge.

There are reports also in rodents suggesting that signals arising from the suprachiasmatic nucleus (SCN), which is considered to be the master circadian clock, affect the hypothalamic neuronal functions such as the regulation of timing of LH surge (reviewed in (53)). In addition, direct neural connections between SCN neurons and GnRH neurons in the hypothalamus have been demonstrated anatomically (54, 55). Finally, the GnRH1 neurons themselves have been reported to express endogenous clock genes (56), which may modify their sensitivity to

various signals by changing the expression levels of certain receptors.

Although previous studies of the diurnal regulatory mechanisms of GnRH1 neurons have been performed using OVX+E mice, I here succeeded in recording, for the first time in vertebrate brains, time-of-day dependent changes in the electrical activity of GnRH1 neurons in the intact brain of daily ovulating medaka without manipulations of the sex steroid milieu. This approach will enable us to use medaka to examine the temporal relationships between the natural ovulatory cycles and GnRH neuronal activity under physiological conditions. Analyzing the contribution of estrous and circadian cyclicity on time-of-day dependent changes in the GnRH neuron activities will be an interesting topic for future research.

### *Pituitary expression of lhb and fshb mRNA also shows time-of-day dependent changes*

I demonstrated that *lhb* and *fshb* mRNA expression in medaka pituitaries showed time-of-day dependent changes, particularly in the primary transcript. Both mRNA and primary transcript for these two genes showed similar time-of-day dependent changes, although *lhb* primary transcript started to change earlier than *lhb* mRNA. Because primary transcripts were measured before splicing, I should in principle be able to use this approach to detect changes in the expression of genes of interest more sensitively than with mRNA. Interestingly, I found a time lag between the peak of *lhb* and *fshb* transcription and the peak of GnRH neuronal activity. This time lag may be due to the effect of GnRH on *lhb* transcription, which took ten hours or more (Fig. 1-4).

In the experiment using isolated pituitaries incubated with GnRH, *lhb* transcription started to increase eight hours after the completion of GnRH peptide treatment and was maintained at increased levels for several hours.

Non-mammalian GnRH receptors, which possess a C-terminal tail that contributes to agonist-dependent receptor desensitization and internalization, show robust agonist-dependent internalization (57). Three subtypes of GnRH receptors are expressed in medaka, and all of them possess a cytoplasmic C-terminal tail (58, 59). Therefore, medaka GnRH receptors are expected to show robust agonist-dependent internalization when they are treated with GnRH. Nonetheless, the increased *lhb* transcription level was maintained for more than 12 hours in my experiments. This prolonged response may be because of *in vitro* incubation of pituitary isolated from the brain; in contrast, *in vivo* the transcription of *lhb* mRNA may be inhibited by other factors such as dopamine, which inhibits LH release in teleosts (60, 61). These results, taken together with my results, suggest that once the LH synthetic pathway has been switched on GnRH receptor internalization itself may not inhibit *lhb* mRNA transcription, and that the effect of agonists may last for ten hours or more.

### *Conclusions*

I propose a working hypothesis concerning temporal regulation of the ovulatory cycle in the brain and pituitary of female medaka (see Fig. 1-5). Activity of GnRH1 neurons increases towards the evening of Day 1 (Fig. 1-2), leading to increased release of GnRH. The released GnRH peptide regulates LH on two different time scales. First, it stimulates the pituitary gonadotropes to release LH on a short time scale (i.e., in several seconds; (39)) thereby causing the LH surge, which then triggers ovulation during the time period before lights-on. At the same time, GnRH facilitates transcription of *lhb* on a slower time scale, ten hours or more (Fig. 1-4); the resulting protein product, LH, will be the source for the next LH surge, on Day2.

Thus, medaka represents an excellent model system for studying the neural mechanisms of control of vertebrate reproduction under the natural conditions, and is expected to provide us with important insights in future work.

## Figure legends

### Figure 1-1

GnRH1 neurons in the ventral POA project directly to the pituitary and express higher level of *gnrh1* mRNA than do dorsal POA GnRH1 neurons. (A, B) Double fluorescence analysis with retrograde labeling of neurons after biocytin injection to the pituitary (green) and GnRH immunohistochemistry (magenta) demonstrates that the GnRH1 neurons of the vPOA project directly to the pituitary (B). White arrowheads in the merged picture indicate neurons that show colocalization of retrograde tracer and mdGnRH peptide. I did not find any colocalization of retrograde labeling (green) and *gnrh1* mRNA (magenta) in the population of dorsal GnRH1 neurons (A). Taken together, I conclude that the ventral population of GnRH1 neurons in POA (vPOA GnRH1 neurons) play a major role in hypophysiotropic function and mainly contribute to the stimulation of gonadotropin release. V, ventricle; IHC, immunohistochemistry. Scale bars, 20 μm. (C) Illustrations of a frontal section and a lateral view (left = rostral) of the medaka brain, showing the plane of section corresponding to panels A, B, and D; note that midline is located in the center of the picture in A. Blue squares indicate the dorsal (D) and ventral (V) populations of GnRH1 neurons shown in A, B, and D. Dm, area dorsalis telencephali pars medialis; Dl, area dorsalis telencephali pars lateralis; Dp, area dorsalis telencephali pars posterior; POm, nucleus preopticus pars magnocellularis; POp, nucleus preopticus pars parvocellularis; OT, tectum opticum; Vp, area ventralis telencephali pars posterior. (D) Representative time-lapse photographs of digoxigenin precipitates visualized for *gnrh1* mRNA detection by *in situ* hybridization in the identical section. Photographs were taken at 10 min, 30

min and 240 min after the application of an alkaline phosphatase substrate, NBT/BCIP. *In situ* hybridization shows two separate populations of *gnrh1* mRNA positive neurons in the area ranging from the telencephalon to the POA. The ventral population of GnRH1 neurons was visualized much earlier during the precipitation, strongly suggesting that the expression of *gnrh1* mRNA is higher in the ventral than the dorsal population. Scale bar, 25 μm.

**Figure 1-2**

Electrical activity of the vPOA GnRH1-GFP neurons in sexually mature female medaka. (A) Enlarged view of the cell bodies of vPOA GnRH1-GFP neurons imaged by conventional fluorescence microscopy. Scale bar, 10 μm. (B) Targeted on-cell patch clamp recording from a vPOA GnRH1-GFP neuron (for 15 seconds). (C, D) Spontaneous firing activities of GnRH1-GFP neurons for one minute in the time period preceding the putative LH surge (at 13:00; C) and just before and during the putative LH surge (at 16:00, D). Bars above the traces indicate the lighting conditions of the aquarium room; light on from 8:00 ~ 22:00 (white boxes) and light off from 22:00 ~ 8:00 (gray boxes). The striped boxes indicate the recording time windows, and the scale bars indicate the action current amplitude and time scale. The table at the bottom of D indicates the recording time groups. (E, F) Comparisons of the mean firing rate (spikes/second; E) and the median instantaneous frequency (Hz; F) indicate that vPOA GnRH1-GFP neurons show higher firing rates and higher median instantaneous frequencies in group e (15:30~19:30, n = 12), compared with group d (11:30 ~ 15:30, n = 11). (G, H) Comparisons of the percentage and the maximum duration of quiescence demonstrate that vPOA GnRH1-GFP neurons show a significantly higher

percentage of quiescent time and a longer duration of quiescence in group d (15:30 ~ 19:30, n = 11), compared with other groups. *, $p < 0.05$ (Tukey-Kramer test) compared to group d.

**Figure 1-3**

Time-of-day dependent changes in the expression of gonadotropin mRNA/primary transcript in the pituitary of sexually mature female medaka. (A) Both *lhb* mRNA and primary transcript show clear time-of-day dependent changes. In *lhb* mRNA, the expression level is highest at 5:00 and remains at relatively low levels between 17:00 and 1:00. This pattern is time-shifted forward for *lhb* primary transcript such that the expression level is highest at 1:00 and 5:00 and remains low between 13:00 and 21:00. (B) Time-of-day dependent changes in levels of *fshb* mRNA are not clear, but levels peak at 5:00 as do those of *lhb* mRNA. *Fshb* primary transcript shows time-of-day dependent changes, and the expression level is highest at 5:00 and remains low between 17:00 and 1:00. The vertical axes of the graphs represent relative expression levels that were normalized relative to the average expression level at 5:00. Tukey-Kramer tests indicate the following levels are statistically significant: a ($p < 0.001$), b ($p<0.01$), and c ($p < 0.05$) are lower compared to 5:00, and d ($p<0.001$), e ($p<0.01$) are lower compared to 1:00.

**Figure 1-4**

GnRH upregulates expression of *lhb* mRNA on a slow time scale in isolated pituitaries *in vitro*. (A) Experimental scheme. Pituitaries were surgically isolated and treated with normal medium (NM) and 10 nM E2 and NM ("without mdGnRH" group), or with 10 nM E2 and 100 nM mdGnRH ("with mdGnRH" group). Two hours

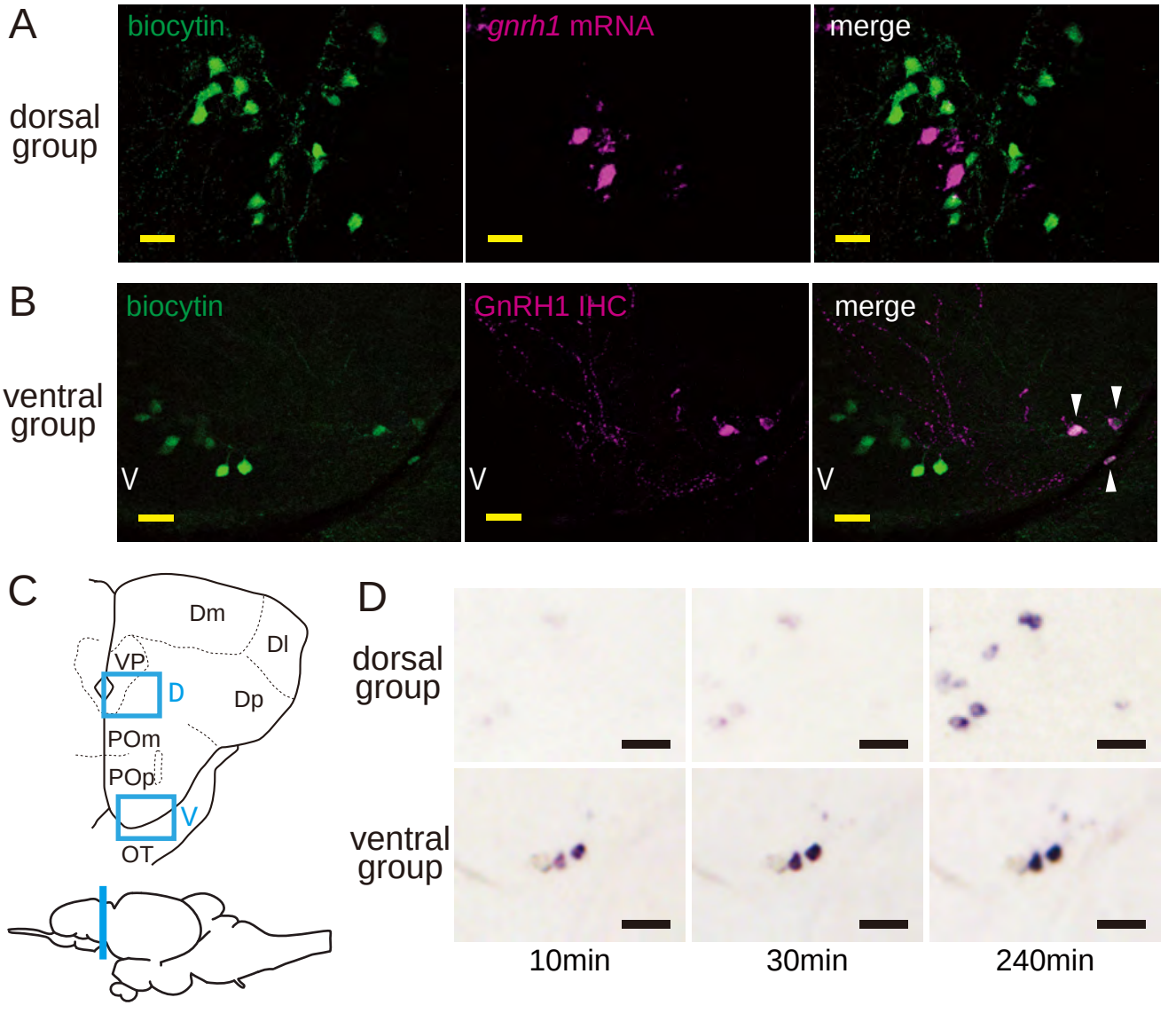
after treatment, pituitaries were washed with NM and incubated in NM for several hours. Pituitaries were collected 0, 4, 8, 12, and 16 hours after the completion of the GnRH or control treatment, and expression of *lhb* mRNA/primary transcript was measured. (B) Relative expression of *lhb* mRNA/primary transcript in the pituitaries was measured using real-time PCR. The level of expression of *lhb* mRNA starts to increase twelve hours after the completion of GnRH treatment, and shows no change in pituitaries not treated with GnRH. Relative expression of *lhb* primary transcript starts to increase more than eight hours after the completion of GnRH treatment. The vertical axes of the graphs represent relative expression levels, normalized by the average expression level measured just after the 2-hour incubation without mdGnRH. Statistically significant differences were obtained using a two-way factorial ANOVA to compare the "with" and "without GnRH" groups (*lhb* mRNA, $p<0.01$; *lhb* primary transcript, NS ($p=0.053$)), to compare each sampling time (*lhb* mRNA, $p<0.01$; *lhb* primary transcript, $p<0.01$), and to examine the interaction effect (*lhb* mRNA, $p<0.01$; *lhb* primary transcript, $p<0.05$). Dunnet's post hoc test, *** $p < 0.001$ and ** $p<0.01$ compared to the level of expression in the "without GnRH" group at time 0 (without GnRH time 0, 4, 8, 12, 16, n= 7, 7, 7, 7, and 5; with GnRH time 0, 4, 8, 12, 16, n=8, 8, 8, 8, and 6, respectively).

**Figure 1-5**

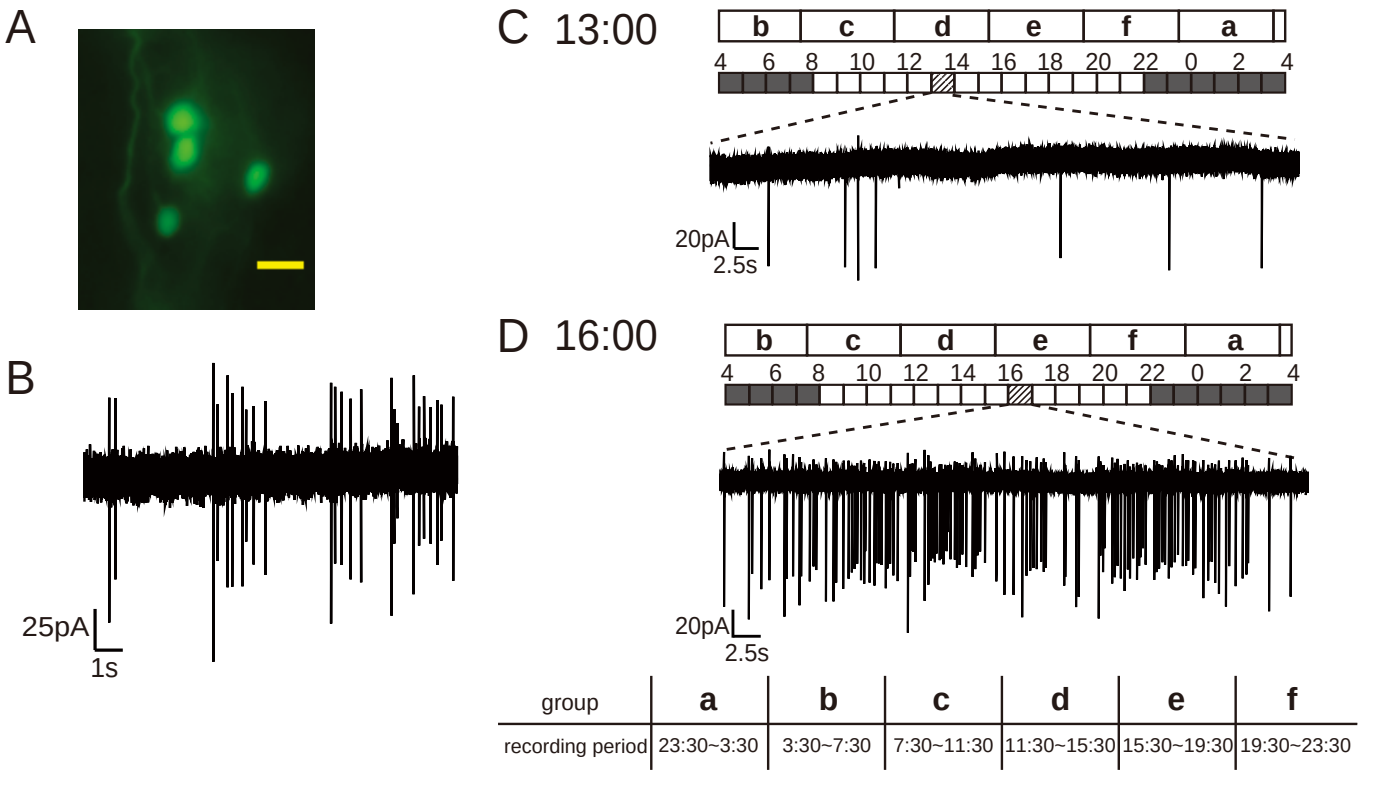
Working hypothesis for the temporal regulation of the ovulatory cycle in the female medaka. Towards the evening of Day1, GnRH1 neuronal activity increases, leading to increased GnRH release. The GnRH peptide stimulates pituitary gonadotropes to release LH within several seconds and causes the LH surge, which triggers ovulation during the night. The GnRH peptide simultaneously acts on the pituitary

gonadotropes to increase the transcription of *lhb* mRNA over the course of several hours, and the synthesized LH will be the source for LH surge on Day2. See text for detail.

# Figure 1-1


A
dorsal group
biocytin
gnrh1 mRNA
merge
B
ventral group
biocytin
GnRH1 IHC
merge
V
V
V
C
Dm
Dl
VP
D
Dp
POm
POp
V
OT
D
dorsal group
ventral group
10min
30min
240min

# Figure 1-2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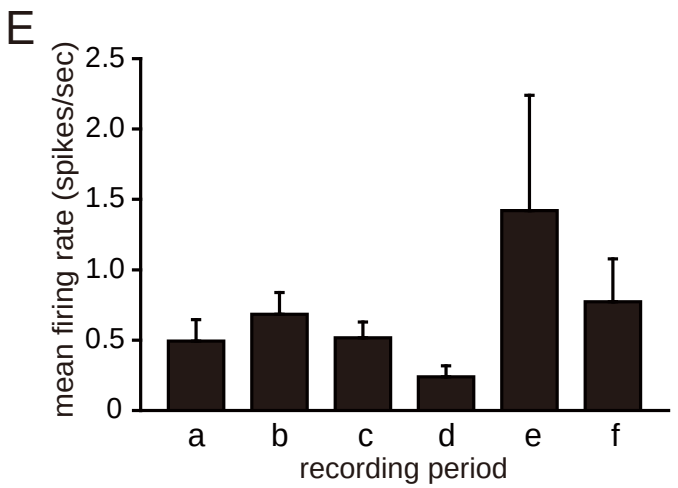
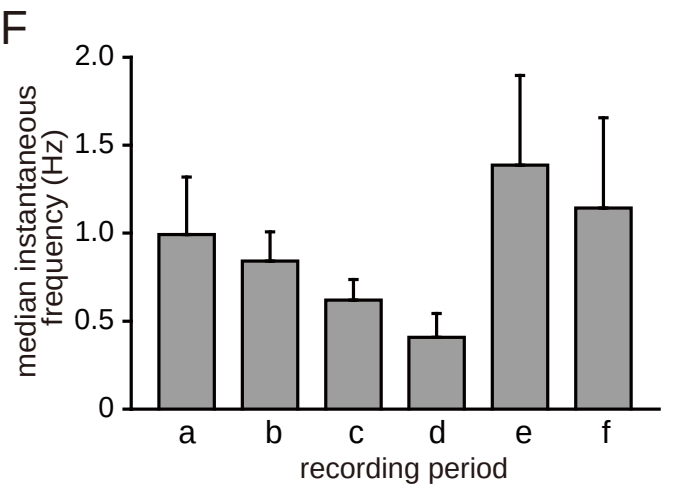
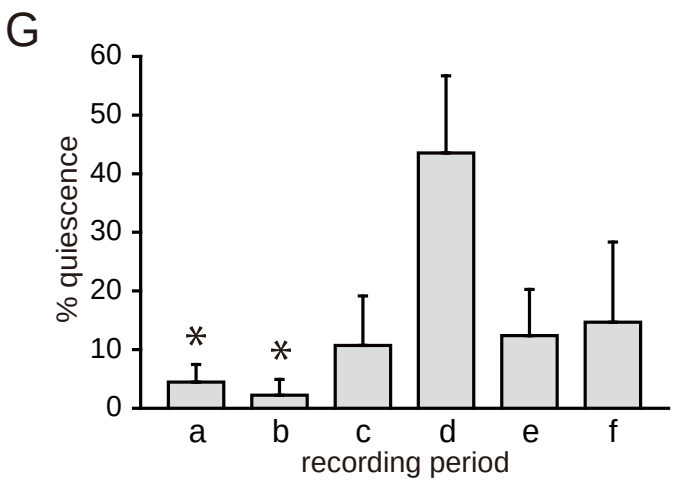
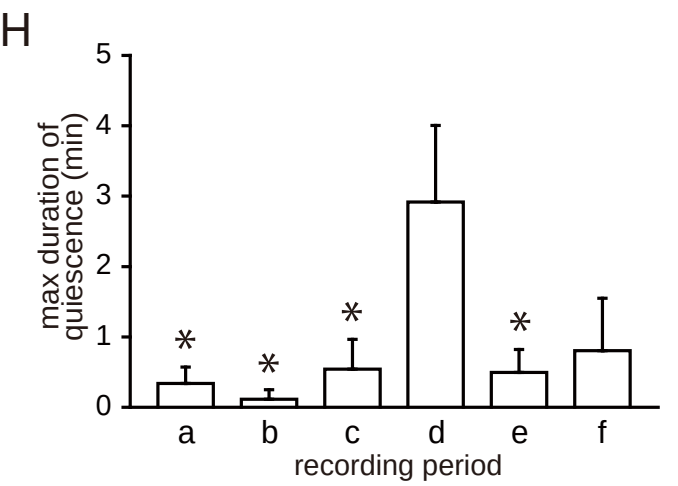
B
25pA
1s
C 13:00
b c d e f a
4 6 8 10 12 14 16 18 20 22 0 2 4
20pA
2.5s
D 16:00
b c d e f a
4 6 8 10 12 14 16 18 20 22 0 2 4
20pA
2.5s
group a b c d e f
recording period 23:30~3:30 3:30~7:30 7:30~11:30 11:30~15:30 15:30~19:30 19:30~23:30

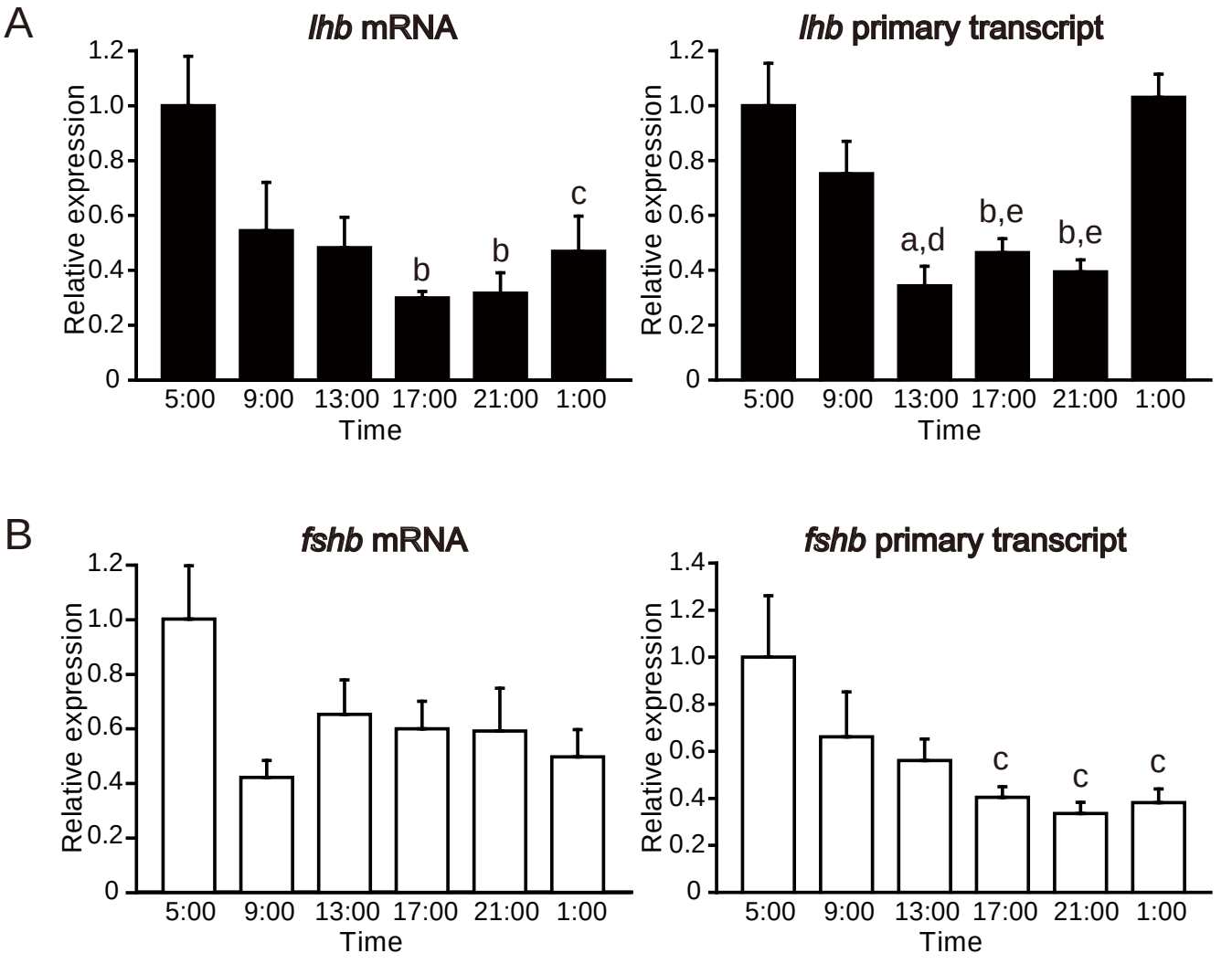

E
mean firing rate (spikes/sec)
0 0.5 1.0 1.5 2.0 2.5
a b c d e f
recording period


F
median instantaneous frequency (Hz)
0 0.5 1.0 1.5 2.0
a b c d e f
recording period


G
% quiescence
0 10 20 30 40 50 60
a b c d e f
recording period


H
max duration of quiescence (min)
0 1 2 3 4 5
a b c d e f
recording period

# Figure 1-3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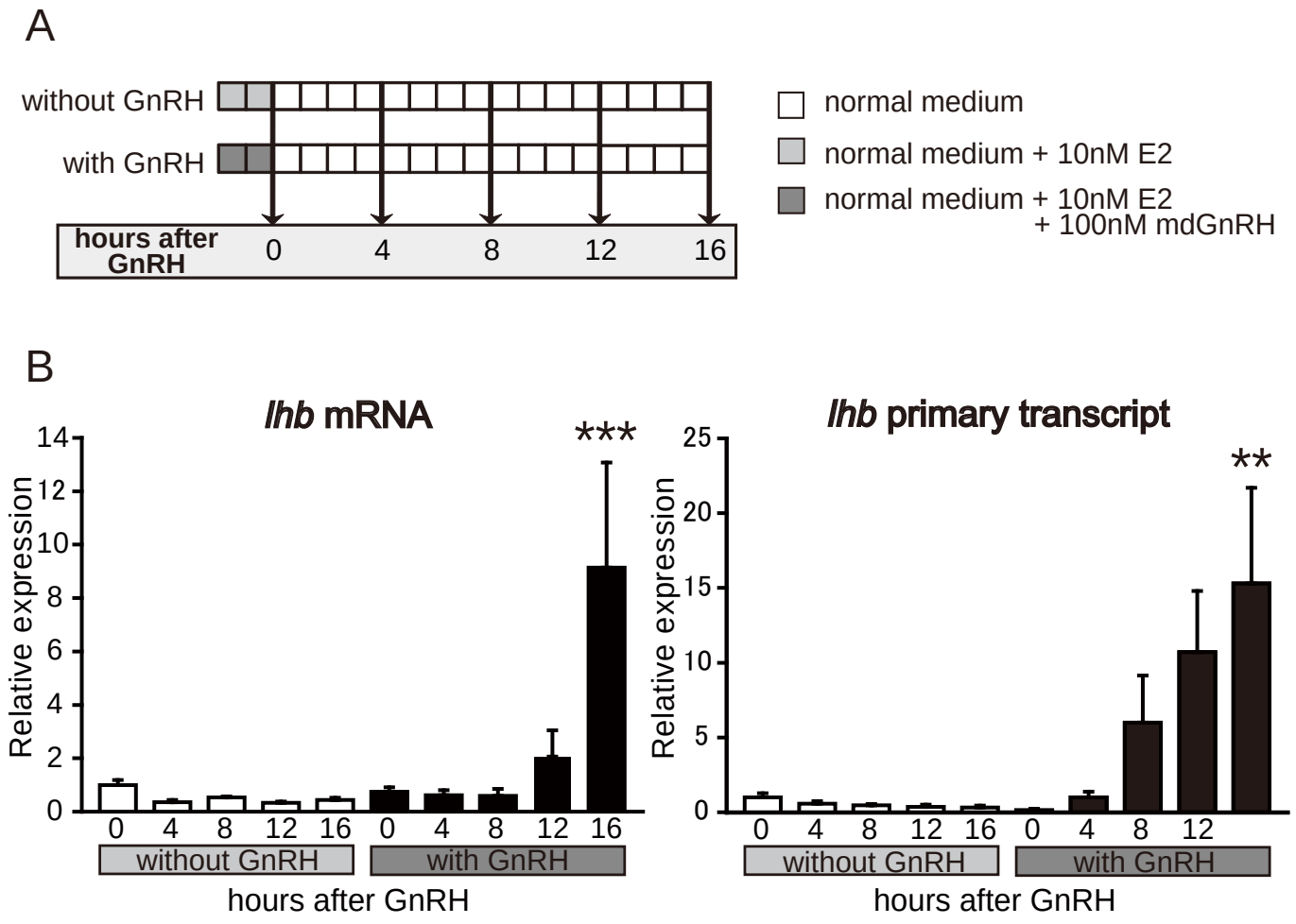
lhb mRNA
lhb primary transcript
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0
b
b
c
a,d
b,e
b,e
5:00
9:00
13:00
17:00
21:00
1:00
Time
B
fshb mRNA
fshb primary transcript
1.4
c
c
c

# Figure 1-4


A
without GnRH
with GnRH
hours after GnRH
0
4
8
12
16
normal medium
normal medium + 10nM E2
normal medium + 10nM E2
+ 100nM mdGnRH
B
lhb mRNA
***
Relative expression
14
12
10
8
6
4
2
0
0 4 8 12 16 0 4 8 12 16
without GnRH
with GnRH
hours after GnRH
lhb primary transcript
**
Relative expression
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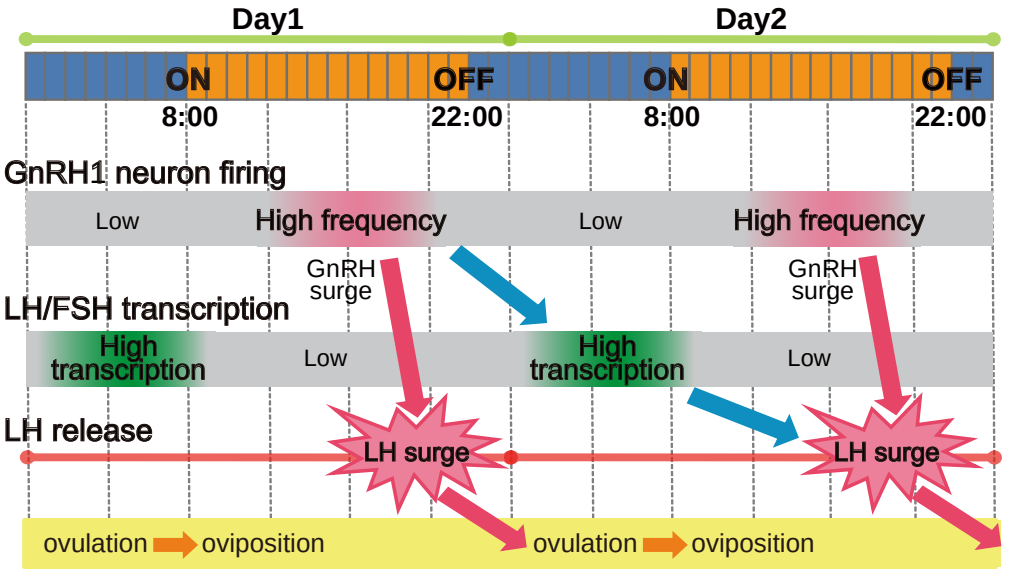
20
15
10
5
0
0 4 8 12 16 0 4 8 12
without GnRH
with GnRH
hours after GnRH

# Figure 1-5


Day1
Day2
ON
OFF
ON
OFF
8:00
22:00
8:00
22:00
GnRH1 neuron firing
Low
High frequency
Low
High frequency
GnRH surge
GnRH surge
LH/FSH transcription
High transcription
Low
High transcription
Low
LH release
LH surge
LH surge
ovulation
oviposition
ovulation
oviposition

## Table 1-1

Primer pairs used for real-time PCR

F: forward, R: reverse

| Usage for | F/R | Nucleotide sequence |
|---|---|---|
| *lhb* mRNA | F | 5'-TGCCTTACCAAGGACCCCTTGATG-3' |
| | R | 5'-AGCTCTCCACAGGGATGCTG-3' |
| *lhb* primary transcript | F | 5'-TAAAGGCTTCAAAGTGGTGCAGTC-3' |
| | R | 5'-TGGAGTGCATGTAAGGGAAGAAC-3' |
| *fshb* mRNA | F | 5'-TGGAGATCTACAGGCGTCGGTAC-3' |
| | R | 5'-AGGGTATGTGACTGACGGATCCAC-3' |
| *fshb* primary transcript | F | 5'-TTTGGAATATTATCCCCCAACCGC-3' |
| | R | 5'-ATGTGGTGAACCCAAAAACTCTGC-3' |
| *actb* mRNA | F | 5'-CCCCACCCAAAGTTTAG-3' |
| | R | 5'-CAACGATGGAGGGAAAGACA-3' |

# Chapter 2

## Physiological Study of GnRH-Induced Differential Regulations of Gonadotropins, LH and FSH: Analysis of $Ca^{2+}$ Dynamics in Transgenic Medaka

## Abstract

Two types of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) are important pituitary hormones for sexual maturation and reproduction, and both of them are centrally regulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. In mammals, these two gonadotropins are secreted from a single type of gonadotropes. The mechanisms of differential regulation by GnRH of the release of two types of gonadotropins with different secretory profiles are still unknown. In teleosts, however, LH and FSH are secreted from separate cellular populations unlike in mammals. This feature makes them useful for studying the regulatory mechanisms of LH and FSH secretions independently. Here, I generated transgenic medaka lines that express $Ca^{2+}$ indicator proteins, inverse-pericam, specifically in the LH or FSH cells. I performed cell-type specific $Ca^{2+}$ imaging of LH and FSH cells, respectively, using the whole brain-pituitary preparations of these transgenic fish in which all neural circuits and GnRH neuronal projection to the pituitary are kept intact. LH and FSH cells showed different $Ca^{2+}$ responses to GnRH. The results suggest differential regulation mechanisms for LH and FSH release by GnRH. Moreover, I also succeeded in detecting the effect on LH cells of endogenous GnRH peptide, which was released by electrical stimulation of the axons of GnRH1 neurons. Thus, my newly-developed experimental model system using the whole brain-pituitary *in vitro* preparation of the transgenic medaka is a powerful tool for analyzing the differential regulatory mechanisms of the release of LH and FSH by multi-synaptic neural inputs to the pituitary.

## Introduction

Pituitary gonadotropins, LH and FSH, are the most important regulators of vertebrate reproduction. In mammals, FSH stimulates the maturation of ovarian follicles, whereas LH induces the follicular rupture, ovulation in female. The synthesis and release of LH and FSH are regulated by GnRH, which is synthesized by neurons in the hypothalamus, GnRH neurons (62). In addition to the gonadotropins, GnRH is suggested to be functionally conserved throughout vertebrates (40). On the basis of accumulating physiological and molecular phylogenic homology data, it is now generally accepted that the two subtypes of teleost gonadotropins, GTH-I and GTH-II, correspond to the tetrapod FSH and LH, respectively (13). Thus, it is suggested that the functions of LH and FSH may have differentiated early in the vertebrate lineage, and the functional and secretory properties of LH and FSH have much in common for each throughout vertebrate species.

The great majority of physiological studies concerning the regulation of gonadotropin release have been performed using mammalian primary cell cultures, particularly from rats (63-66). However, there are some inevitable difficulties in studying the differential regulation mechanisms of gonadotropin release in mammals because of the fact that each mammalian gonadotrope produces both LH and FSH. By contrast, teleost is a good model to overcome some difficulties of mammalian preparations and to study the neural control of the adenohypophysis because of their unique characteristics. First, LH and FSH are secreted from distinct populations of LH and FSH cells that express those hormones separately (reviewed in (13) and as for medaka in (30)). Second, the adenohypophysis is

directly innervated by neurosecretory fibers, and is not intercalated by portal vessels (14). Third, the teleost pituitary is highly compartmentalized, and the same types of cells are clustered (13). Also, especially in medaka, their pituitary is small and transparent enough to keep them healthy and to observe their fluorescence of $Ca^{2+}$ indicators or fluorescent proteins from outside of the whole brain-pituitary preparations without dispersing or slicing.

To take advantage of such features, I here generated a transgenic medaka that expresses a genetically encoded calcium indicator, inverse-pericam (IP) (67), in LH and FSH cells for the first time in vertebrates to study differential release mechanisms of those hormones at the cellular level. IP is a genetically encoded calcium indicator that shows stronger fluorescence at lower $Ca^{2+}$ concentrations and weaker fluorescence at higher $Ca^{2+}$ concentrations, which is the inverse of the general $Ca^{2+}$ indicators. I chose IP to make it easier to identify the labeled cells at the resting low $Ca^{2+}$ concentrations, which helped the screening process. As the GnRH-induced $Ca^{2+}$ rise in gonadotropes has been reported to be accompanied by an increase in the membrane capacitance (surface membrane area) of gonadotropes (65, 66), $Ca^{2+}$ rise could be considered as the indicator of the exocytotic release of hormones from the gonadotropes.

By using these transgenic medaka lines, I succeeded, for the first time, in analyzing the stimulatory effects of GnRH separately on the LH and FSH cells at the cellular level. Furthermore, in the present study, I used whole brain-pituitary *in vitro* preparations to keep the neural circuit to the pituitary and the networks inside the pituitary intact by taking advantage of the small and transparent brain and pituitary of a small teleost fish, medaka. I succeeded in analyzing GnRH-induced $Ca^{2+}$ changes differentially in the individual LH and FSH cell by

using the whole brain-pituitary preparations. Moreover, I also succeeded in detecting the effect of endogenous GnRH peptide on LH cells, which was released by electrical stimulation of the axons of GnRH1 neurons. These results suggest that my newly developed experimental model system using the transgenic medaka lines is a powerful tool for analyzing the differential regulatory mechanisms of the release of LH and FSH by neural inputs to the pituitary.

## Materials and Methods

### *Animals*

Male and female wild type drR medaka and all transgenic medaka (*lhb:IP*, *fshb:IP*, *gnrh1:EGFP* (39), and *gnrh1:EGFP* x *lhb:IP*) were maintained in pairs under a 14-h light and 10-h dark photoperiod (light on at 8:00 and light off at 22:00) at water temperature of 27 °C. I used sexually matured female and male medaka (body weight: female 261±14 mg, male 240±18 mg; gonadosomatic index (GSI): 7.27±0.35 % in female) that spawned for at least three consecutive days. I did not find any significant sexual differences in results, and I therefore combined the data from both sexes. The fish were fed two to three times a day with live brine shrimp and flake food. All animals were maintained and used in accordance with the guidelines of the University of Tokyo for the use and care of experimental animals.

### *Generation of constructs for transgenic medaka*

The medaka *lhb* and *fshb* loci were isolated by screening a bacterial artificial chromosome (BAC) library (*lhb*: ola1-108H20, *fshb*: ola1-110G16). The primer pairs used for promoter PCR amplification were as follows; for LHβ promoter, Forward:

5'- AAAAATCAGCGAGAGTCGCTGGTT -3', and Reverse: 5'-TGTGAAAAATAAAATTGACTC -3', for FSHβ promoter, Forward: 5'-ACATCCATTGGCCTCTGTAAATGA -3', and Reverse: 5'-CCTCTGCCTGGTGCAGTACC -3'. The fragment containing the 5'-flanking region (about 2.5 kb) along with exon 1 of *lhb* or *fshb* was PCR amplified from the screened BAC clone. In each gene, exon 1 coded a 5'-untranslated region, and the initiation methionine codon was present at the 5' end of exon 2. Each fragment was fused with the inverse-pericam-coding sequence (67) followed by the polyadenylation signal of the bovine growth hormone (BGH) gene and subcloned into the cloning vector pGEM-T (Promega, Madison, WI) (Fig. 2-2 A). These constructs were purified and dissolved in 10 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA.

*Generation of transgenic medaka lines*

DNA constructs were diluted into 10~50ng/μL with PBS and 0.02% phenol red and injected into the cytoplasm of one- or two-cell-stage embryos (F0). The injected embryos were intercrossed to identify the germ line founders. Individual adults from positive pairs were then outcrossed to identify the individual founder fish. Heterozygous transgene carriers in the F1 generation were identified by their presence of fluorescence in the embryos. To obtain homozygous transgenic offsprings, the carriers were crossed with each other. In the present study, I used homozygous progeny.

*Histology*

The pituitary was fixed by 4% paraformaldehyde in PBS. The fixed pituitary was frontally cryo-sectioned at 30 μm using a cryostat (CM 3050S, Leica microsystems,

Wetzlar, Germany) and mounted onto MAS-GP typeA coated glass slides (Matsunami, Osaka, Japan) as described before (30). For double labeling immunohistochemistry (IHC) for LH or FSH cells and GnRH fibers in the pituitary, I used *gnrh1:EGFP* transgenic medaka line that was generated by Ms. Akiko Takahashi (The University of Tokyo). The GnRH fibers were visualized by anti-EGFP antiserum (68) (diluted 1:1000 with PBST) and AlexaFluor488 conjugated anti-rabbit IgG (diluted 1:800 with PBST; Invitrogen, Carlsbad, CA). LH and FSH cells were visualized by anti-medaka LHβ antiserum or anti-medaka FSHβ antiserum (diluted 1:10000 with PBST) (69) and AlexaFluor555 conjugated anti-mouse IgG (diluted 1:800 with PBST; Invitrogen). For double labeling of *lhb* or *fshb* mRNA and IHC for inverse-pericam, I used *lhb:IP* or *fshb:IP* transgenic medaka. *Lhb* or *fshb* mRNA were visualized with a standard protocol described in (30) using DIG-labeled *lhb* or *fshb* probe. After ISH probe hybridization, the sections were incubated with anti-EGFP antiserum (diluted 1:1000 with DIG-1 buffer), rinsed twice with DIG-1 buffer, and incubated with TSA (tyramide signal amplification)-plus biotin reagent (PerkinElmer) for 30 min, rinsed with DIG-1 buffer twice, and incubated with ABC reagents (1% A solution and 1% B solution in DIG-1 buffer, Vector, Burlingame, CA) for 1 h. The sections were rinsed twice with DIG-1 buffer, incubated with Alexa Fluor 488-conjugated streptavidin (diluted 1:500 with DIG-1 buffer, Invitrogen) and alkaline phosphatase-conjugated anti-DIG antibody (diluted 1:1000 with DIG-1 buffer, Roche) for 2 h. Then, the sections were rinsed twice with DIG-1 buffer. After the EGFP signals were detected, the alkaline phosphatase activity, which was used to label *lhb* or *fshb* mRNA, was detected using Fast-Red substrate kit (abcam, Cambridge, UK) according to the manufacturer's instructions. The incubation for this substrate was carried out until

visible signals were detected and was stopped by washing in PBS containing 0.5 mM EDTA. Then, the sections were coverslipped with CC/Mount (Diagnostic BioSystems, Pleasanton, CA). The fluorescence was observed under confocal laser-scanning microscope LSM-710 (Carl Zeiss, Oberkochen, Germany) at 1-μm optical section. I also observed the whole brain-pituitary preparations using MVX10 stereoscopic microscope (Olympus, Tokyo, Japan).

## *$Ca^{2+}$ imaging*

### *Preparation and intracellular $Ca^{2+}$ imaging with Fura-2*

The fish were anesthetized by immersion in tricaine methanesulfonate (MS-222; dissolved 0.02% in tap water) and then decapitated. The brain was dissected out with intact pituitary and kept in fish artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 134, KCl 2.9, $CaCl_2$ 2.1, $MgCl_2$ 1.2, HEPES 10, and glucose 15 (adjusted to pH 7.4 with NaOH). I prepared Fura-2 solution (ACSF with 10 μM Fura2-AM (DOJINDO, Kumamoto, Japan) and 0.02% CremophorEL (Fluka, St. Louis, MO), vortexed it for 10 seconds and sonicated it for 4 seconds on ice using a sonicator (MS-50, HEAT SYSTEMS-ULTRASONICS, INC., Farmingdale, NY). After spinning down, the whole brain-pituitary preparation was incubated in Fura-2-AM solution for 30~60 min at room temperature to load Fura-2 in the cytoplasm of pituitary cells. After the incubation, the preparation was washed with ACSF and placed in a hand-made chamber filled with ACSF in the ventral side-up direction (Fig. 2-1B). The preparation was perfused with ACSF at a continuous flow rate (2mL/min). Fura-2 fluorescence were detected by the Chroma 74000 filter set (D340×, D380× exciter; 505DCLP dichroic mirror; HQ535/50m emitter) housed in the Lambda DG-4 Xenon light source, excitation filter exchanger (Sutter

Instruments, Novato, CA), and the BX-51WI upright epifluorescence microscope (Olympus, Tokyo, Japan). The fluorescence was recorded (exposure: 100 ms; interval: 5 sec) using QuantEM 512SC EMCCD camera (Photometrics, Tucson, AZ) and the Metafluor imaging software (Molecular Devices Corp., Downingtown, PA). For Fura-2, the intensity ratio of emission (at 510 nm) from the alternating 340 and 380 nm excitation was monitored.

### *Preparation and $Ca^{2+}$ imaging with inverse-pericam*

The whole brain-pituitary preparation from *lhb:IP* or *fshb:IP* transgenic medaka was prepared as described above. For the detection of IP fluorescence, I replaced the excitation filter in the Lambda DG-4 Xenon light source and excitation filter exchanger for FF01-474/23-25 (Semrock, Rochester, NY). Positions of the LH and FSH cells were visually identified under epifluorescence illumination, and fluorescence images of IP were recorded (exposure: 50 ms; interval: 5 sec). In all experiments using repetitive mdGnRH applications (Fig. 2-5), the interval (washout time) between the 1st and the 2nd trial was 10 minutes, and that between the 2nd and the 3rd one was 20~30 minutes. The mdGnRH applications under various antagonists in Fig. 2-5 were performed 10 minutes after pretreatment with antagonists.

### *GnRH response of LH cells evoked by electrical axonal stimulation of GnRH neurons*

For this category of experiments, I used double-transgenic medaka (*gnrh1:EGFP* crossed with *lhb:IP*) to label both GnRH1 fibers and LH cells. I crossed homozygous transgenic offsprings of *gnrh1:EGFP* and *lhb:IP* and obtained double heterozygous *gnrh1:EGFP* x *lhb:IP* transgenic medaka. I prepared the whole brain-pituitary

preparation as described above. I used the same filter set as that for IP imaging. First, I set the focus on the bundle of GnRH1 fibers that run rostrocaudally in the hypothalamus and project to the pituitary (Fig. 2-6A, B). For the stimulation electrode, I used a mono-polar electrode (an electrolytically polished stainless steel insect pin, 1 μm in tip diameter, which was lacquer-coated, leaving a 50-100μm uncoated bared tip). I attached the mono-polar hand-made stimulation electrode to the EGFP-labeled GnRH1 fiber bundle unilaterally under the fluorescent microscope. After attaching the stimulation electrode, I performed $Ca^{2+}$ imaging of the LH cell layer, while stimulating the GnRH1 fiber bundle (500-μs pulses at +250 μA every 20 ms for 20 trains) via an electronic stimulator equipped with an isolator (SEN-3301 and SS-302J, NIHON KOHDEN, Tokyo, Japan).

### *Drugs*

The medaka GnRH (mdGnRH) peptide (pE-H-W-S-F-G-L-S-P-G-$NH_2$) was synthesized chemically by Sigma-Aldrich Japan (Tokyo, Japan). I used 100 nM mdGnRH for the co-application with $CdCl_2$ or 2APB (Fig. 2-5C,D,G,H) and 50 nM GnRH for other experiments with *lhb:IP* and *fshb:IP*. Analog M ($_D$-pE-$_D$-F-$_D$-W-S-Y-$_D$-W-L-R-P-G-$NH_2$), a competitive GnRH receptor antagonist (70), was purchased from Sigma-Aldrich (L6524, St. Louis, MO) or synthesized by GL Biochem (Shanghai, China). Both mdGnRH and analog M were dissolved in 100 μM pure water and diluted by ACSF to an optimal concentration. 2-Aminoethoxydiphenyl borate (2APB), an $IP_3$ receptor antagonist, was dissolved by dimethyl sulfoxide (DMSO) in 100 mM and diluted by ACSF.

### *Data analysis*

All values are shown as mean ± SEM. In all imaging experiments, the time when drug reached the experimental chamber was defined as Frame 0. $F_0$ was calculated as an average of five frames, Frame -8 to -4. The fluorescence intensity change ($\Delta F/F_0$) was calculated as $(F_0-F)/F_0$, and the peak $\Delta F/F_0$ was picked up from 25 frames, Frame -3 to 21 in *lhb:IP*, and from 50 frames, Frame -3 to 46 for *fshb:IP*. All Fura2 experiments were analyzed in the same paradigm for *lhb:IP*, using the ratio of emission intensity (340 nm/380 nm; R) instead of the IP fluorescence intensity, and the $\Delta R$ values were calculated as $R-R_0$, where $R_0$ is the averaged ratio of the Frame -8 to -4. In the rise time analysis (Fig. 2-3E), I calculated $(F_0-F)/F_0$ for each cell (LH; 27~36 cells and FSH; 22~32 cells from one experiment), drew averaged trace for each experiment, and compared the half rise time from the onset to the peak of GnRH responses among the averaged traces (n=7). The $Ca^{2+}$ fluctuation events were identified with the event detection algorithm in TaroTools (Igor macro set written by Dr. Taro Ishikawa, Jikei University School of Medicine) (Fig. 2-4). The peak detection procedure for this algorism is as follows. First, the raw data was linearly approximated for defining the baseline level. Next, the time points when the difference between the baseline and the raw trace is larger than the threshold value were picked up chronologically. Then, the maximum value of the raw trace that is found just before the first point when the polarity of the backward derivative of the 3-point moving averaged trace changed from positive to negative, were defined as a peak. For the $Ca^{2+}$ peak detection, I used 0.001 as the threshold value. For the detection of simultaneous $Ca^{2+}$ peaks, I selected all fluorescent pituitary cells found from one focus plane and detected $Ca^{2+}$ peaks from each cell and plotted the $Ca^{2+}$ peak events as a raster plot (Fig. 2-4B, E). I calculated and plotted percentage of cells that showed $Ca^{2+}$ peaks at each time point (gray line in Fig. 2-4C,

F). I detected peaks from 3-point moving averaged trace (black line in Fig. 2-4C, F) with the same algorithms used for the $Ca^{2+}$ peak detection using 3% as the threshold value (Fig. 2-4C, F).

Mann-Whitney *U* tests were performed for the comparison between the peak $\Delta F/F_0$ of vehicle and GnRH application, and that between 50% rise time of LH and FSH cells. Steel's multiple comparison tests were performed for repetitive GnRH application experiments, compared to the 1st GnRH application. These statistical analyses were performed using Kyplot5 (Kyence, Tokyo, Japan). Significance levels were set as $P < 0.05$. All the imaging data were analyzed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD) with MBF ImageJ plug-ins (Tony Collins, McMaster University, Ontario, Canada) and Igor Pro 6.22A (WaveMetrics Inc., Lake Oswego, OR) with TaroTools.

## Results

### *GnRH-induced $Ca^{2+}$ rise in pituitary cells*

To test the usefulness of the whole brain-pituitary preparations for $Ca^{2+}$ imaging, I first examined $Ca^{2+}$ response induced by GnRH application in unidentified pituitary cells. I loaded Fura2-AM to the entire pituitary and recorded $Ca^{2+}$ response induced by GnRH. Generally, the pituitary of teleosts consists of various cell types arranged in characteristic clusters, and the LH and FSH cells are separated in different clusters in the pituitary (13, 30). FSH cells were localized in the dorsal proximal pars distalis (PPD), and LH cells in the ventral PPD (shown in Fig. 2-1A as a schematic illustration, see (30, 71) for detailed distribution). I set the focus at the surface of PPD, the presumptive LH cell area (white dotted square in

Fig. 2-1A). I used a synthetic medaka GnRH (mdGnRH) peptide, which is suggested to be the intrinsic GnRH peptide secreted by hypophysiotropic GnRH1 neurons (40), and the mdGnRH application induced transient $Ca^{2+}$ rise in the pituitary cells (Fig. 2-1 C). The peak amplitude (Δratio of F340/F380) of $Ca^{2+}$ response showed dose dependent changes (1 nM: 0.0080±0.0029, 10 nM: 0.0065±0.0025, 30 nM: 0.0935±0.0057, 100 nM: 0.1471±0.0049, 300 nM: 0.1614±0.0114, 1000 nM: 0.2266±0.0110; n= 55 cells [from 3 fish], 94 [3], 101 [3], 377 [16], 78 [3], 99 [3], respectively; Fig. 2-1 D, E).

### *Generation of lhb:IP and fshb:IP transgenic medaka*

In medaka, GnRH1 neurons in the ventral preoptic area (vPOA) directly project to the pituitary (39). I performed double immunohistochemistry (IHC) for LH or FSH and GnRH (EGFP-immunoreactive in *gnrh1:EGFP* transgenic medaka) and found that GnRH1 fibers project to both LH and FSH cell layers in the pituitary (Fig. 2-2 D, E). To examine the GnRH effect on each gonadotrope (LH cell or FSH cell) separately, I generated transgenic medaka lines that specifically express IP, a genetically encoded calcium indicator, in either LH or FSH cells. I generated transgenic lines using plasmid-based DNA constructs that contain 5' flanking region of *lhb* gene (2.3kb) or *fshb* gene (2.2kb) immediately upstream of IP sequence (Fig. 2-2A). I injected *lhb*:IP construct to 380 fertilized eggs and *fshb*:IP construct to 330 fertilized eggs. I crossed the F0 generation fish and succeeded in obtaining one homozygous transgenic line for each construct. I examined the specificity of IP expression in the pituitary using double labeling of *lhb* or *fshb* mRNA by ISH and IP by EGFP IHC. I found that both of these transgenic lines showed specific expression of inverse-pericam in LH or FSH cells (Fig. 2-2B, C). Therefore, I performed further

experiments using these transgenic lines.

*Different $Ca^{2+}$ responses are induced by mdGnRH in LH and FSH cell*

I performed cell-type specific $Ca^{2+}$ imaging of individual LH and FSH cells respectively, using these transgenic lines as the whole brain-pituitary preparations. In the LH cells, mdGnRH application induced a rapid and transient $Ca^{2+}$ rise ($\Delta F/F_0$ in vehicle: 0.0107±0.0008, mdGnRH: 0.0258±0.0013; $P < 0.001$; n=118 [4]; Fig. 2-3A, C). On the other hand, the FSH cells showed a slow and long lasting $Ca^{2+}$ responses in response to mdGnRH, in comparison with those in LH cells ($\Delta F/F_0$ in vehicle: 0.0164±0.0017, mdGnRH: 0.0474±0.0025; $P < 0.001$; n=139 [5]; Fig. 2-3, B and D). The half rise time for GnRH responses was compared between LH and FSH cells. The FSH cells showed a significantly longer rise time compared with LH cells (half rise time in LH cells: 20.0±3.36 sec, FSH cells: 49.2±3.16 sec; $P < 0.001$; n=7 averaged traces for each; Fig. 2-3E). In some preparations, I could observe spontaneous $Ca^{2+}$ responses in LH and FSH cells (Fig. 2-4A, D). Among them, the LH cells tended to show simultaneous $Ca^{2+}$ rise more frequently compared with the FSH cells (Fig. 2-4B, C). Their simultaneous events occurred within 30 to 60 second periods. The FSH cells, on the other hand, tended to show less simultaneous events compared with that of LH cells (Fig. 2-4E, F). Interestingly, the FSH cells also showed short-interval spontaneous $Ca^{2+}$ rises in individual cells (Fig. 2-4D), whereas there was no such pattern in the LH cells. In either LH or FSH cells, I did not observe characteristic GnRH-induced $Ca^{2+}$ oscillations, which have been reported for the rat gonadotropes (65). I examined the effects of analog M, a GnRH receptor antagonist (70), on the spontaneous $Ca^{2+}$ fluctuations in my preparation, but they were not completely diminished in either LH or FSH cells, even under high

analog M concentrations, in which neither LH nor FSH cells responded to exogenous application of mdGnRH peptide (data not shown).

*GnRH receptor-mediated $Ca^{2+}$ rise is mainly derived from the intracellular store*

To examine the source of $Ca^{2+}$ rise, I then performed experiments using various pharmacological agents. I first confirmed that three repetitive applications of GnRH did not significantly change the $Ca^{2+}$ response amplitude in the identical preparation in both LH and FSH cells ($\Delta F/F_0$ in LH cells: 1st GnRH: 0.0286±0.0012, 2nd GnRH: 0.0285±0.0012 ($P$ = 0.9979), 3rd GnRH 0.0281±0.0011 ($P$ = 0.9424), n=105 [3], Fig. 2-5A; FSH cells: 1st GnRH: 0.0491±0.0029, 2nd GnRH: 0.0465±0.0031 ($P$ = 0.7836), 3rd GnRH 0.0468±0.0035 ($P$ = 0.8305), n=120 [4], Fig. 2-5E). Then, I compared the amplitude of GnRH responses in the presence of various receptor antagonists. First, I examined the effect of analog M to confirm that the GnRH-induced $Ca^{2+}$ rise in LH and FSH cells is a GnRH receptor-mediated response. The mdGnRH responses in the presence of 5 μM analog M were severely diminished in both LH and FSH cells ($\Delta F/F_0$ in LH cells: 1st GnRH: 0.0270±0.0011, 2nd GnRH+analog M: 0.0075±0.0006 ($P$ < 0.001), 3rd GnRH 0.0226±0.0006 ($P$ < 0.001), n=135 [4], Fig. 2-5B; FSH cells: 1st GnRH:0.0308±0.0023, 2nd GnRH+analog M: 0.0112±0.0009 ($P$ < 0.001), 3rd GnRH 0.0218±0.0017 ($P$ < 0.001), n=96 [3], Fig. 2-5F), indicating that the responses are mediated by GnRH receptors. To determine whether GnRH-induced $Ca^{2+}$ rise derived from the extracellular $Ca^{2+}$ influx or $Ca^{2+}$ release from the intracellular store, I next examined the GnRH effect in the presence of $Cd^{2+}$, a broad-spectrum voltage gated $Ca^{2+}$ channel blocker. 100 μM $CdCl_2$ did not change the amplitude of mdGnRH response ($\Delta F/F_0$ in LH cells: 1st GnRH: 0.0208±0.0009, 2nd GnRH+$CdCl_2$: 0.0236±0.0010 ($P$ = 0.0753), 3rd GnRH

0.0233±0.00010 ($P$ = 0.1248), n=85 [3], Fig. 2-5C; FSH cells: 1st GnRH: 0.0593±0.0038, 2nd GnRH+$CdCl_2$: 0.0560±0.0031 ($P$ = 0.7147), 3rd GnRH 0.0497±0.0032 ($P$ = 0.0827), n=125 [3], Fig. 2-5G). As GnRH induces the release of $Ca^{2+}$ from an inositol 1,4,5-triphosphate ($IP_3$)-sensitive store in the rat gonadotrope (66), I used 2-APB, an $IP_3$ receptor antagonist. 100 μM 2APB significantly decreased the amplitude of GnRH-induced $Ca^{2+}$ rise in both LH and FSH cells ($\Delta F/F_0$ in LH cells: 1st GnRH: 0.0135±0.0007, 2nd GnRH+analog M: 0.0084±0.0005 ($P$ < 0.001), 3rd GnRH 0.0134±0.0005 ($P$ = 0.9819), n=78 [3], Fig. 2-5D; FSH cells: 1st GnRH: 0.0667±0.0043, 2nd GnRH+2APB: 0.0459±0.0034 ($P$ < 0.001), 3rd GnRH 0.0582±0.0035 ($P$ = 0.1997), n=109 [3], Fig. 2-5H).

### *$Ca^{2+}$ rise in LH cells in response to the electrical axonal stimulation of GnRH1 neurons can be visualized*

In the previous experiments, I stimulated gonadotropes using exogenous applications of GnRH peptide. By taking advantage of the whole brain-pituitary preparation, I next examined $Ca^{2+}$ responses of LH cells by the electrical axonal stimulation of GnRH1 neurons. In medaka, vPOA GnRH1 neurons directly project to the pituitary by forming thick axon bundles (Fig. 2-6A and B). I used the *gnrh1:EGFP* x *lhb:IP* double transgenic medaka to visualize the GnRH1 axon bundles under the fluorescence microscopy. I recorded $Ca^{2+}$ fluctuations from the LH cells while electrically stimulating unilateral GnRH1 fiber bundle with a mono-polar electrode placed on it (Fig. 2-6B). The electrical stimulation (500-μs pulses at +250 μA every 20ms for 20 trains) induced a $Ca^{2+}$ rise in LH cells (Fig. 2-6C), the time course of which was similar to that induced by exogenous mdGnRH application (Fig. 2-3A). The averaged value of peak amplitude ($\Delta F/F_0$) was 0.0118±0.0007, and the average time to peak after stimulation was 52.3±2.27

seconds (n= 224 cells in 18 trial from 10 fish). Prior analog M treatment (5 μM) severely diminished the electrical stimulation-induced $Ca^{2+}$ rise in LH cells (Fig. 2-6 C,D; $\Delta F/F_0$ in LH cells: 1st Stim.: 0.0071±0.0005, 2nd Stim.+analog M: 0.0023±0.0004 ($P < 0.001$), 3rd Stim. 0.0546±0.0005 ($P < 0.05$), n=33 [3], Fig. 2-6C, D). As the sensitivity to the GnRH peptide is different among individuals, I only analyzed data from the stimulation experiment with a complete set of three stimulations.

## Discussion

### *GnRH-induced $Ca^{2+}$ rise in LH and FSH cells can be visualized using the whole brain-pituitary preparation*

GnRH was first found as a hypothalamic peptide that stimulates LH release. Just as the name indicates, GnRH has also been shown to stimulate FSH release in addition to LH release (72, 73). However, the mechanisms and properties of FSH release are not well understood in contrast to the great deal of knowledge in LH release. One of the reasons may be that LH and FSH are co-released from a single gonadotrope in mammals. To take advantage of the teleost pituitaries where LH and FSH cells are completely separate, I generated transgenic medaka lines that express a genetically encoded calcium indicator, IP, specifically in the FSH or the LH cells. Until now $Ca^{2+}$ responses in LH cells (not the mammalian gonadotropes which co-express LH and FSH in the same cells) have been studied mainly in goldfish using Fura-2 or Indo-1 (31, 74-76), but there has been no report on the FSH cells. This is probably because of the difficulty of identifying FSH cells from the goldfish dispersed cell culture, because they distinguished the LH cells from the other pituitary cells only by their morphological features (77). The newly generated

transgenic medaka lines in the present Chapter allow us to identify LH and FSH cells by their fluorescence and to record cell type-specific $Ca^{2+}$ responses separately using the whole brain-pituitary preparations in which the neural circuit and the pituitary cell network are kept intact.

GnRH-induced LH release and $Ca^{2+}$ rise in gonadotropes have been well known from the studies in '90s using pituitary cell culture (63-66). GnRH-induced $Ca^{2+}$ rise in the gonadotrope is accompanied by an increase in membrane capacitance, which indicates the occurrence of exocytosis (63-66). This suggests that GnRH-induced $Ca^{2+}$ rise can be an index of the timing of hormone exocytosis from the gonadotropes. LH cells begin to respond to mdGnRH from around 30 nM in my Fura2 experiments (Fig. 2-1D, E). This concentration is rather higher than the other studies in the literature (around 0.01 nM in rat (slice) (78), 1 nM in goldfish and medaka (culture) (76, 79), 0.1 nM in catfish (culture) (80)). The difference seems to arise from the difference in the preparations. As the cells are not directly exposed to the solution in my whole brain-pituitary preparations, they need higher concentration than the other studies. However, I succeeded in detecting small but sufficient $Ca^{2+}$ rise in LH cells by the electrical stimulation of GnRH1 neurons, which is expected to induce small amount of intrinsic GnRH release locally in the pituitary. Therefore, the effective concentration of 30 nM in the present experiment should be considered as a physiological concentration, and the present experiments are considered to represent what occurs *in vivo*. Moreover, the time course and the wave forms of $Ca^{2+}$ response were similar to those induced in goldfish and African catfish LH cells in dispersed pituitary cell culture (74-76, 80). Note that examination of Fura2 based measurements in *lhb*:IP medaka failed to detect responses to GnRH in IP negative cells at least adjacent cells of IP positive cells. These results are consistent with the

fact that no report has shown the surface cells in adenohypophysis but LH cells express GnRH receptors.

In medaka, mdGnRH induced rapid, transient and non-oscillatory $Ca^{2+}$ rise in LH cells. Previous studies in mice, rats and goldfish showed that GnRH induces $Ca^{2+}$ responses in gonadotropes, which are broadly divided into two categories: oscillatory and non-oscillatory responses (64, 65, 76, 78). Unlike these studies, I only observed non-oscillatory $Ca^{2+}$ rise in medaka LH cells, which has also been reported in catfish (80). The GnRH response curve obtained in medaka LH cells in the present study appears to be similar to the non-oscillatory response curve in mice (78).

I demonstrated for the first time that FSH cells also show GnRH receptor-mediated $Ca^{2+}$ rise. In contrast to the LH cells, the GnRH-induced $Ca^{2+}$ rise in FSH cells are slow and long-lasting. The responses in both LH and FSH cells are mediated by GnRH receptors, but their response curves were different from each other. There are some possible explanations. One is that both LH and FSH cells express GnRH receptors, but the difference in signaling cascade downstream of GnRH receptor activation may cause the difference in $Ca^{2+}$ signaling. From the results of tilapia pituitary cell culture, it was suggested that *lhb* and *gpa* or *fshb* transcription were regulated by different signaling cascades downstream of GnRH receptor activation. *lhb* and *gpa* transcription were regulated by GnRH receptor-mediated signals coupled to both PKC-PKA and MAPK pathways, but FSH transcription was mainly regulated by PKA-CREB-CRE pathway (13, 81). However, this applies to the transcription but not the hormone release, and different signaling cascades may be recruited and cause the difference in GnRH-induced $Ca^{2+}$ rise in LH or FSH cells. One of the other possibilities for the difference between LH

and FSH cells is presence/absence or the subtype expression of the regulators of G protein signaling (RGS) protein coupled to GnRH receptors. The RGS proteins interact with active Gα subunits and accelerate their intrinsic GTPase activity of Gα subunits leading to their deactivation and termination of downstream signals. The RGS proteins modify the amplitude and the time course of G protein signaling, including $Ca^{2+}$ signaling, differently according to their subtypes (82). For example, RGS8 modulates both activation and deactivation of $G\alpha_{q/11}$-coupled receptors specifically and in a splicing variant-type dependent manner (83). At least, two family members, RGS3 and RGS10, are implicated in the regulation of GnRH receptor coupling (84). Therefore, LH and FSH cells may have the same signaling cascades that activate $IP_3$-induced $Ca^{2+}$ release, but the time course of G $G\alpha_{q/11}$ subunit activation may be different because of the difference in the subtype of RGS proteins expressed in the LH and FSH cells. Medaka has three kinds of GnRH receptors, all of which are expressed in the pituitary and coupled with $G\alpha_{q/11}$ signaling pathway (58). However, their distribution in the pituitary and the existence of signaling cascades other than $G\alpha_{q/11}$ are still unknown. Therefore, LH and FSH cells may express different subtype of GnRH receptors, which may cause the difference in $Ca^{2+}$ responses. Further studies should reveal differential regulation of LH and FSH cells.

The present results showed that the main source of GnRH-induced $Ca^{2+}$ rise in both LH and FSH cells is the $IP_3$-sensitive intracellular $Ca^{2+}$ store (see Fig. 2-5). Although the specificity of 2APB has not been reported to be high enough as a blocker of intracellular $Ca^{2+}$ release, the combined results of my experiments using $CdCl_2$ to block the voltage-dependent influx should argue against the importance of $Ca^{2+}$ influx. The GnRH signaling in gonadotropes has been well-studied in rat (65,

66). These studies showed that GnRH-induced $Ca^{2+}$ rise did not depend on the extracellular $Ca^{2+}$ but the $IP_3$-sensitive $Ca^{2+}$ stores. Both the $Ca^{2+}$ rise and the hormone release (increase in membrane capacitance) occur in $Ca^{2+}$-free solution (65). In teleosts, however, there are many contradictory results even in the same species. GnRH-induced gonadotropin release were attenuated by the use of $Ca^{2+}$-deficient medium or voltage gated $Ca^{2+}$ channel inhibitors (tilapia (85); snakehead (86); goldfish (87)). Concerning the GnRH-induced $Ca^{2+}$ rise in goldfish gonadotropes, extracellular $Ca^{2+}$ was important for GnRH-induced $Ca^{2+}$ rise (31), but in another study the authors showed that extracellular $Ca^{2+}$ deprivation did not affect GnRH-induced $Ca^{2+}$ rise (76). Johnson *et al.* (76), showed different results of the effect of extracellular $Ca^{2+}$ on the GnRH-induced $Ca^{2+}$ rise; treatment of $Ca^{2+}$-deficient medium or voltage-gated $Ca^{2+}$ channel blockers for a long time may have depleted the intracellular $Ca^{2+}$ store and changed the balance of $Ca^{2+}$ homeostasis. They concluded that GnRH induced $Ca^{2+}$ rise derived from the intracellular store but not from the extracellular $Ca^{2+}$ influx in goldfish LH cells. In this respect, my results are in agreement with this goldfish study and the mammalian studies, and suggest the existence of similar signaling cascades in medaka LH cells. Recently, Strandabø *et al.* (79) reported on the LH cell-specific $Ca^{2+}$ responses to GnRH2 peptide in a dissociated pituitary culture, using Fura-2 imaging with *lhb:EGFP* transgenic medaka. They observed both monophasic and biphasic $Ca^{2+}$ changes evoked by GnRH2 and suggested that the intracellular $Ca^{2+}$ mainly contributes to the first peak, and the extracellular $Ca^{2+}$ contributes to the secondary peak. This apparent discrepancy between the results of Strandabø *et al.* (79) and my present study might be because of the difference in the molecular species of GnRH peptide used for the experiments, preparations, or properties of the

$Ca^{2+}$ indicators. GnRH1 (present result) and GnRH2 (79), may induce different responses, because it has been reported that the signaling cascades to induce $Ca^{2+}$ rise in goldfish dispersed LH cells are different between responses to GnRH2 and those to GnRH3 (reviewed in (74)). I performed GnRH2 peptide application experiments with LH and FSH transgenic fish, but I did not find significant difference in the shape of $Ca^{2+}$ responses between GnRH1 (mdGnRH) and GnRH2 (cGnRH-II) peptides (data not shown). Although I did not perform statistical analysis, the $Ca^{2+}$ responses to GnRH2 peptide tended to be stronger than to GnRH1 peptide. Okubo *et al.* demonstrated that GnRH2 peptide activates all three GnRH receptors more strongly than GnRH1 peptide, and my results are consistent with theirs (58, 59). Furthermore, Strandabø *et al.* used dissociated pituitary cells cultured for 2-7 days after dissociation and were loaded with Fura2-AM. The long-term cell culture may have changed the characteristics of the cells from those of *in vivo* pituitary cells. Other possibilities may include differences in binding and dissociation properties of each calcium indicator, Fura2 and inverse-pericam.

### *Detection of neural input-induced $Ca^{2+}$ rise in LH cells*

In the present study, I observed $Ca^{2+}$ rise in LH cells induced by electrical stimulation of the unilateral GnRH1 axon bundle projecting to the pituitary. The shape and the time course of the response induced by the stimulation were similar to those by exogenous GnRH application, and the $Ca^{2+}$ rise was diminished by GnRH receptor antagonist. These results indicate that electrical stimulation effectively evoked GnRH1 release from the axon terminals of the GnRH1 neurons in the pituitary and that the present preparation is sensitive enough to detect intrinsic GnRH-induced $Ca^{2+}$ rises in the LH cells. It may be noted that the amplitude of the

$Ca^{2+}$ responses induced by electrical stimulation was smaller than that by mdGnRH application (compare with Fig. 2-3C and Fig. 2-6D). There are several possibilities for this difference in the response amplitude. First, it is possible that stronger stimulation was needed to release substantial amount of GnRH peptide from the terminals of GnRH1 neurons. To avoid direct electrical stimulation of LH cells, however, I unfortunately could not use stimulation stronger than +250 μA. Second, I used unilateral electrical stimulation to the GnRH fiber bundle, which may have stimulated only some of the GnRH fibers to result in much weaker $Ca^{2+}$ responses compared with the bath application of GnRH solution. In any case, I here demonstrated for the first time that GnRH neurons actually release GnRH peptides in the pituitary to stimulate the gonadotropes. Furthermore, these results indicate that my transgenic models can be used to analyze not only the direct exogenous input to gonadotropes but also the inputs that are mediated by neural activity in the brain. Next, it should be interesting to reveal the relationship between the electrical activity pattern of GnRH1 neurons and LH release.

I also observed spontaneous $Ca^{2+}$ fluctuations in both LH and FSH cells (Fig. 2-4). LH cells in some preparations showed synchronized small $Ca^{2+}$ fluctuations, which may indicate synchronized neural input to the LH cells or electrical synchronization among the LH cells. The FSH cells showed sufficiently large spontaneous responses compared to the responses stimulated by mdGnRH. Spontaneous $Ca^{2+}$ fluctuation tended to be reduced under analog M treatment but did not completely diminished in both LH and FSH cells (data not shown). This suggests that LH and FSH cells receive inputs from unidentified neurons in addition to GnRH neurons. There are some examples that also suggest the importance of cellular integrations in the pituitary. For example, gonadotropes and

somatotropes are reported to regulate gonadotropin and GH release and transcription each other via autocrine/paracrine release of gonadotropin and GH in grass carp (88). This means that dissociation of pituitary cells may have changed their environment, leading to a change in their gene expression patterns or their activities. Therefore, it is important to keep the networks in the pituitary almost intact for precise analysis. In this respect, my transgenic model should be a powerful tool to study the neural and humoral regulatory mechanisms of LH and FSH release in detail by taking advantage of the whole brain-pituitary *in vitro* preparation.

In summary, I generated transgenic medaka lines that express $Ca^{2+}$ indicators specifically in LH or FSH cells and succeeded for the first time in recording different patterns of $Ca^{2+}$ rises triggered by GnRH in LH and FSH cells separately. This is a remarkable experimental system which allows us to maintain the axonal projections of the GnRH neurons to the pituitary intact by taking advantage of the features of a small teleost brain and pituitary. This model system allows us to analyze the multi-synaptic neuronal circuitry regulating the pituitary functions. Particularly, I should be able to analyze the regulatory mechanisms of FSH release, which has been less intensively studied to date. It should be an exciting in the future to study the neuroendocrine mechanisms of HPG axis regulation, which may include novel regulators of gonadotropin secretion, especially FSH.

## Figure legends

### Figure 2-1

$Ca^{2+}$ imaging from the whole brain-pituitary preparation using Fura2.

(A) A schematic illustration of medaka pituitary. Left, the whole pituitary seen from the ventral side. The area of gonadotropes is shown in purple. Right, frontal section of the pituitary. The LH and FSH cell layers are shown in pink and blue, respectively. The dotted white square indicates the recording area in Fura2 imaging. (B) Schematic illustration of the experimental setup for $Ca^{2+}$ imaging. (C) Representative $Ca^{2+}$ response to 100 nM mdGnRH from unidentified pituitary cells. Each trace from 15 cells is shown in thin gray lines, and averaged trace from 15 cells is shown in a thick black line. (D) Representative dose responses from an identical preparation. (E) Dose response relationship between mdGnRH peptide concentration and the maximum amplitudes of the $Ca^{2+}$ rise. R, rostral; C, caudal; D, dorsal; V, ventral

### Figure 2-2

Generation of transgenic medaka lines.

(A) The gene constructs for the transgenic medaka expressing inverse-pericam (IP) under the control of *lhb* or *fshb* promoters. (B, C) LHβ and FSHβ cell-specific expressions of IP in *lhb:IP* and *fshb:IP* transgenic medaka, respectively. The left pictures show the ventral view of the pituitary of *lhb:IP* and *fshb:IP* transgenic medaka. The white dashed lines indicate the boundaries of the pituitaries. (Scale bar: 50 μm) The right pictures show the frontal sections of the pituitary. Magenta, *lhb/fshb* mRNA ISH; green, EGFP IHC for IP labeling. (Scale bar: 25 μm) (D)

Double IHC for LHβ (magenta) and EGFP (green) in the pituitary of *gnrh1:EGFP* transgenic medaka. The white dashed lines indicate the boundaries of the pituitaries. (Scale bar: 20 μm) (E) Double IHC for FSHβ (magenta) and EGFP (green) in the pituitary of *gnrh1:EGFP* transgenic medaka. (Scale bar: 20 μm) R, rostral; C, caudal

**Figure 2-3**

LH cells and FSH cells show different $Ca^{2+}$ responses to mdGnRH.

(A,B) Representative traces of $Ca^{2+}$ responses to mdGnRH in *lhb:IP* or *fshb:IP* transgenic medaka. The right graphs show traces from 10 cells (thin gray lines) and averaged trace (thick black line). The left pictures show the analyzed cells (the red circles indicate ROI) corresponding to the right graphs. (Scale bar: 50 μm) Moving average was applied to the traces for every three points. (C, D) The peak amplitudes of responses to vehicle and 50 nM mdGnRH applications in LH cells (C) and FSH cells (D). (E) Time to 50% rise in response to 50 nM mdGnRH application in LH and FSH cells. The LH cells showed significantly quicker $Ca^{2+}$ rise than the FSH cells. ***, $P < 0.001$. R, rostral; C, caudal

**Figure 2-4**

Spontaneous $Ca^{2+}$ fluctuations in LH and FSH cells.

(A-C) Representative data for spontaneous $Ca^{2+}$ fluctuations in 23 LH cells, indicating simultaneous oscillation of spontaneous $Ca^{2+}$ fluctuations among them. (A) Representative spontaneous traces from the same recording. The detected $Ca^{2+}$ peaks were marked by asterisks. (B) Raster plot of the timing of $Ca^{2+}$ peaks. The shadowed areas correspond to the area including 5 seconds before and after the

peak time detected in (C). (C) Percentage of cells showing $Ca^{2+}$ rise events simultaneously (gray: raw trace, black: 3-point moving averaged trace). Simultaneous $Ca^{2+}$ peaks detected by the analysis are marked by asterisks. (D-F) Representative data for spontaneous $Ca^{2+}$ fluctuations in 35 FSH cells similar to those in (A-C). Asterisks indicate the peaks detected by analysis.

**Figure 2-5**

GnRH receptor mediated-$Ca^{2+}$ rise is derived from $IP_3$-sensitive $Ca^{2+}$ store and not from extracellular $Ca^{2+}$ influx in both LH cells and FSH cells.

(A, E) The peak amplitude in repetitive three 50 nM mdGnRH applications in LH cells (A) and FSH cells (E). (B, F) The peak amplitude in 50 nM mdGnRH during 5μM analog M application in LH cells (B) and FSH cells (F). (C, G) The peak amplitude in 100 nM mdGnRH during 100μM $CdCl_2$ application in LH cells (C) and FSH cells (G). (D, H) The peak amplitude in 100 nM mdGnRH during 100 μM 2APB application in LH cells (D) and FSH cells (H). ***, $P < 0.001$.

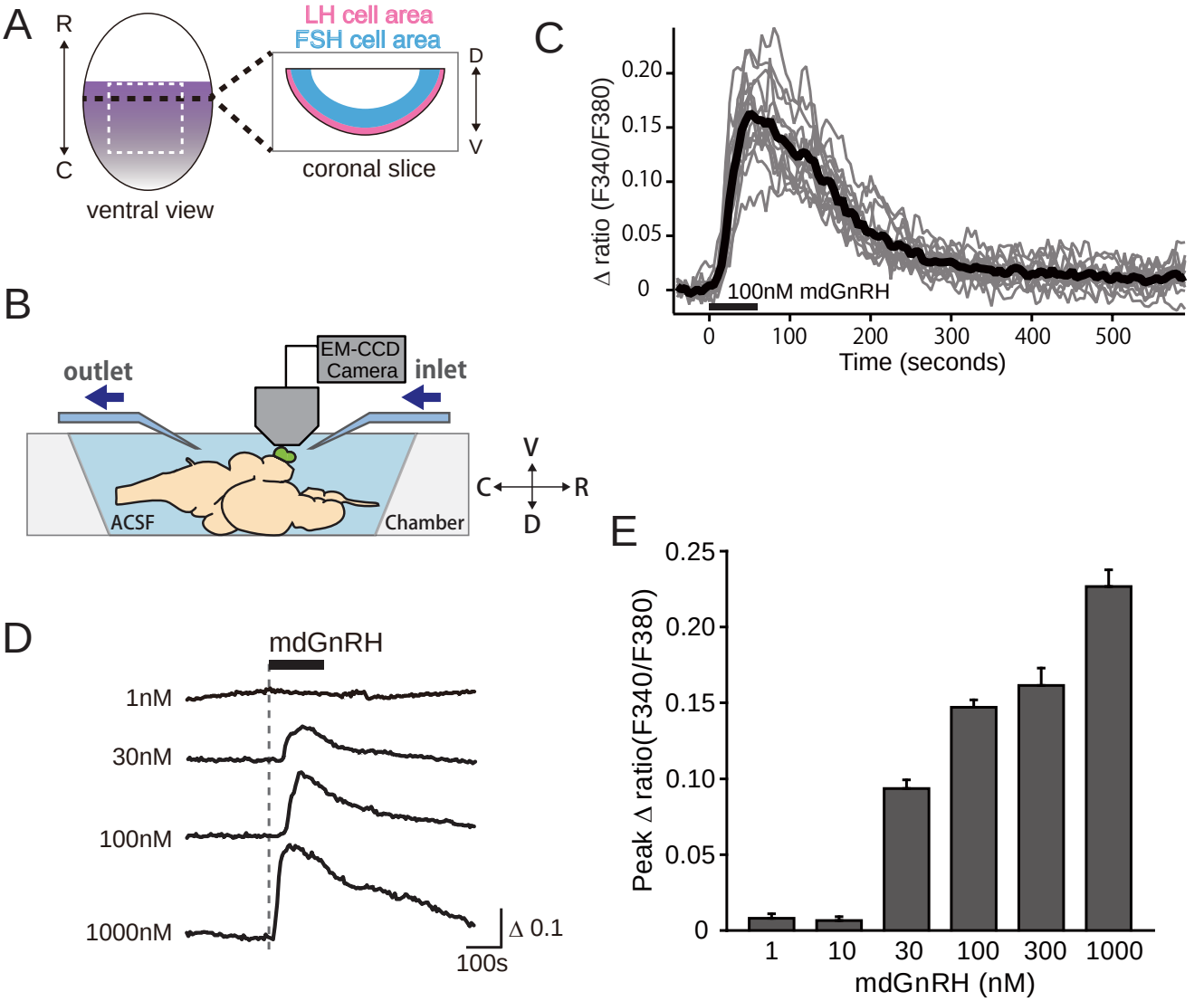
**Figure 2-6**

Release of intrinsic GnRH by stimulating GnRH neuron axons induces $Ca^{2+}$ rise in LH cells.

(A) Morphological evidence that vPOA GnRH1 neurons directly project to the LH cells in the pituitary. (a) Schematic illustration of medaka whole brain from the ventral side. The blue square in (a) is magnified in (b). (b) Enlarged view of the POA and pituitary of *gnrh1*:EGFP transgenic medaka, indicating that GnRH1 fibers (white arrowheads) directly project to the pituitary LH cell area (green fibers in the pituitary). (c) Magnified view of the pituitary in (b). For the axonal stimulation of

GnRH1 neurons, I placed a stimulation electrode on the bundle of *gnrh1:EGFP* axons at the yellow arrowhead. (B) Schematic illustration of the experimental setup for the electrical stimulation and $Ca^{2+}$ imaging. I placed the electrode (black triangle) right on the entry of the GnRH1 fiber bundle into the pituitary. The pink area in the pituitary indicates the LH cell area. I recorded $Ca^{2+}$ response from PPD (gray dashed square on the right figure). (C) Representative example of $Ca^{2+}$ responses after electrical axonal stimulation of GnRH1 neurons. The arrows indicate the timing of electrical stimulation. Each graph shows traces from 12 cells (thin gray line) and an averaged trace (thick black line). The moving average was applied to the traces for every three points. (D) The peak amplitude in three times of repetitive GnRH1 fiber stimulations in the LH cells. Five μM analog M was used as an antagonist for GnRH receptor. ***, $P < 0.001$ and *, $P < 0.05$.

# Figure 2-1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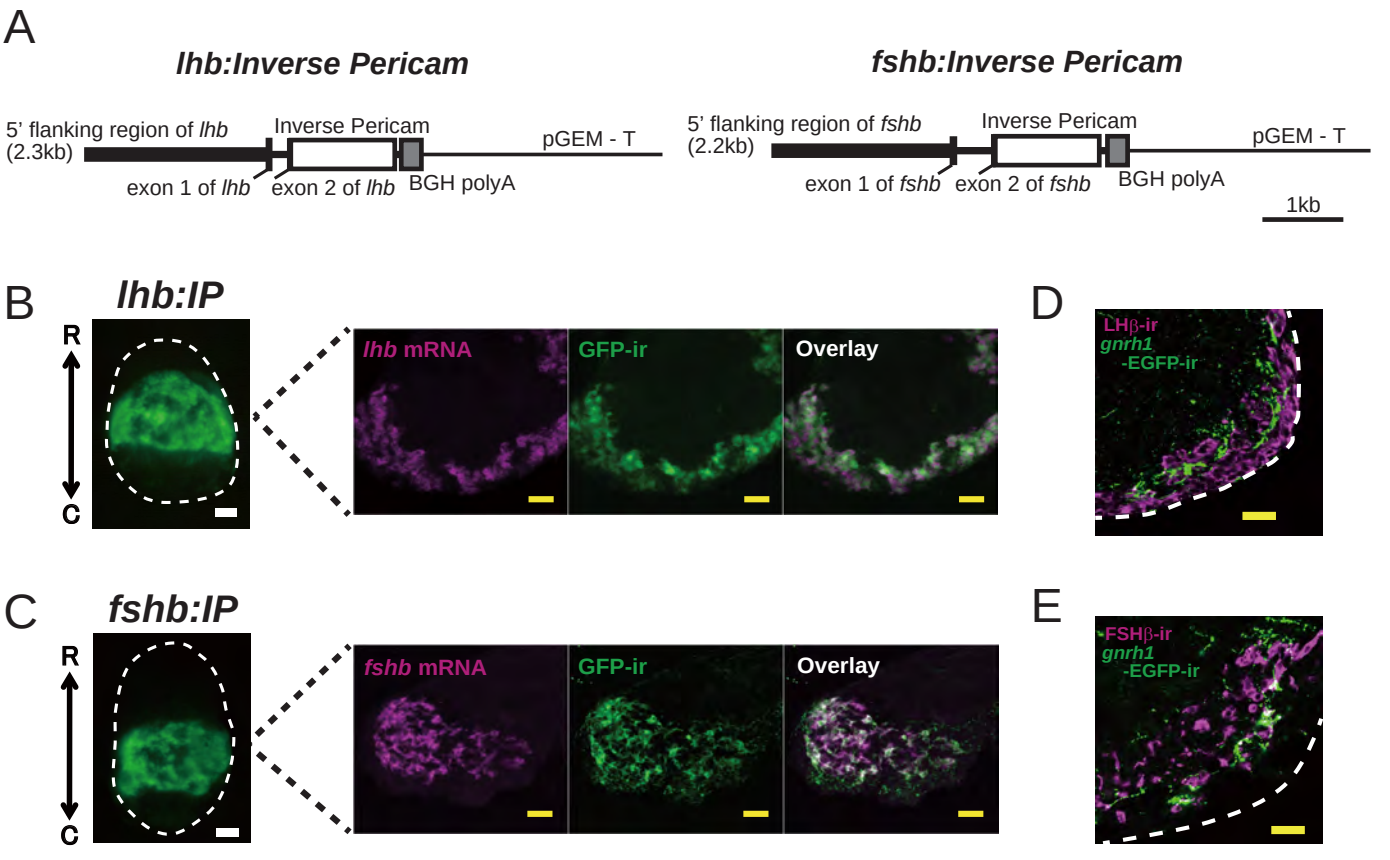
R
C
ventral view
LH cell area
FSH cell area
D
V
coronal slice
B
outlet
EM-CCD Camera
inlet
ACSF
Chamber
V
C
R
D
C
Δ ratio (F340/F380)
0.20
0.15
0.10
0.05
0
100nM mdGnRH
0
100
200
300
400
500
Time (seconds)
D
mdGnRH
1nM
30nM
100nM
1000nM
Δ 0.1
100s
E
Peak Δ ratio(F340/F380)
0.25
0.20
0.15
0.10
0.05
0
1
10
30
100
300
1000
mdGnRH (nM)

# Figure 2-2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Inverse Pericam
pGEM - T
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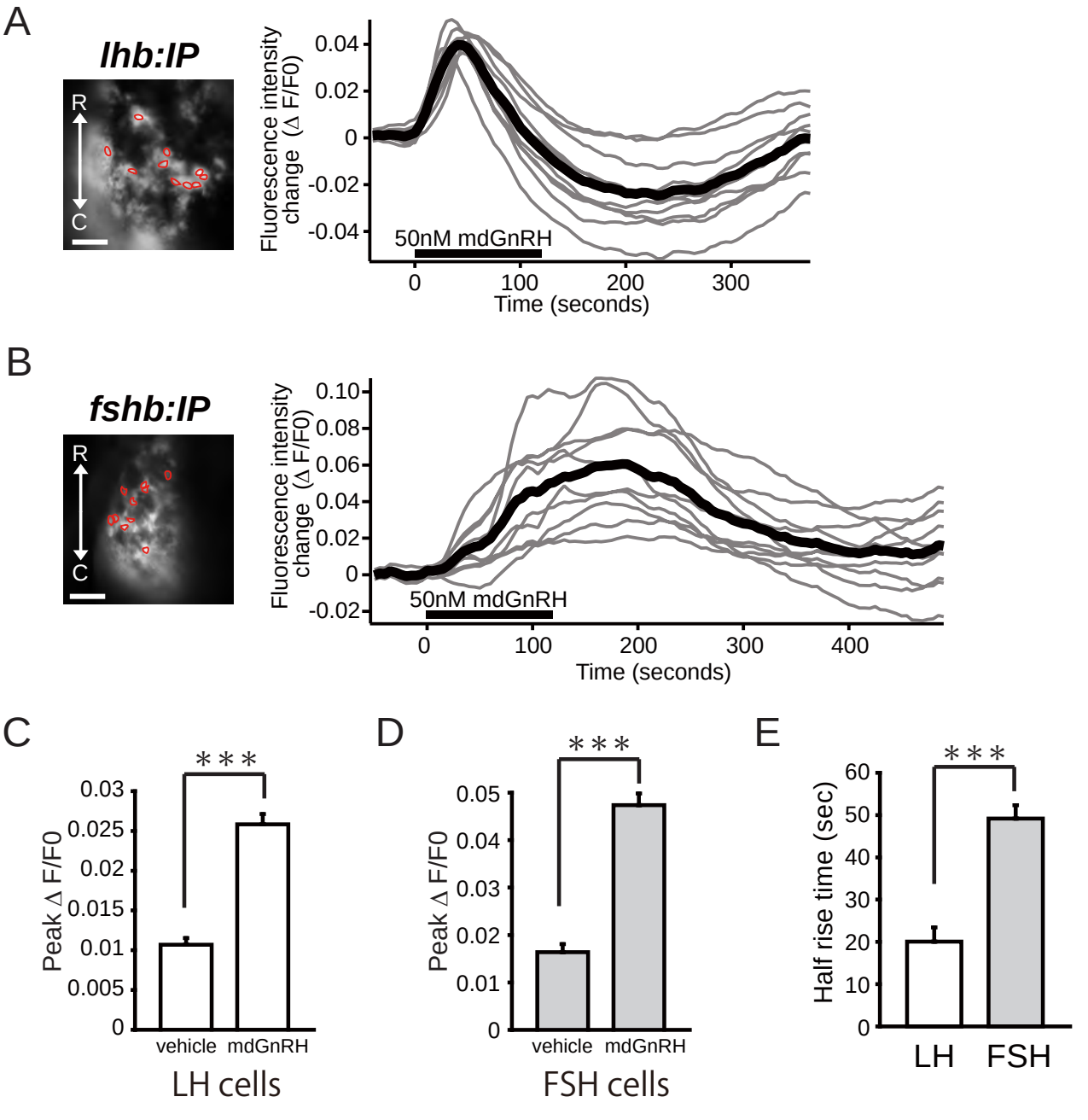
exon 2 of lhb
BGH polyA
fshb:Inverse Pericam
5' flanking region of fshb
(2.2kb)
Inverse Pericam
pGEM - T
exon 1 of fshb
exon 2 of fshb
BGH polyA
1kb
B
lhb:IP
R
C
lhb mRNA
GFP-ir
Overlay
C
fshb:IP
R
C
fshb mRNA
GFP-ir
Overlay
D
LHβ-ir
gnrh1
-EGFP-ir
E
FSHβ-ir
gnrh1
-EGFP-ir

# Figure 2-3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lhb:IP
R
C
Fluorescence intensity change (Δ F/F0)
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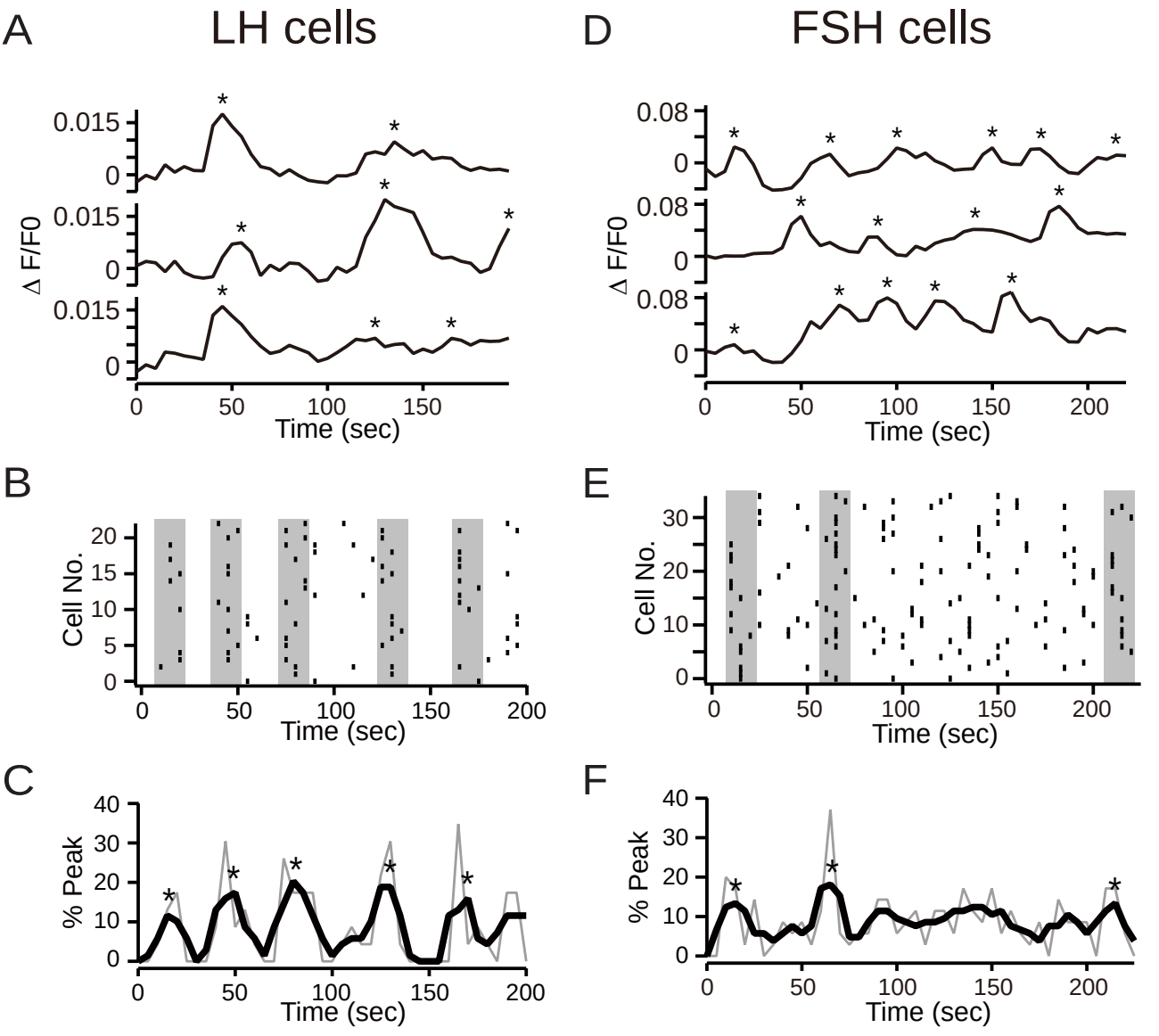
0.02
0
-0.02
-0.04
50nM mdGnRH
0
100
200
300
Time (seconds)
B
fshb:IP
R
C
Fluorescence intensity change (Δ F/F0)
0.10
0.08
0.06
0.04
0.02
0
-0.02
50nM mdGnRH
0
100
200
300
400
Time (seconds)
C
***
Peak Δ F/F0
0.03
0.025
0.02
0.015
0.01
0.005
0
vehicle
mdGnRH
LH cells
D
***
Peak Δ F/F0
0.05
0.04
0.03
0.02
0.01
0
vehicle
mdGnRH
FSH cells
E
***
Half rise time (sec)
60
50
40
30
20
10
0
LH
FSH

Figure 2-4


A
LH cells
D
FSH cells
Δ F/F0
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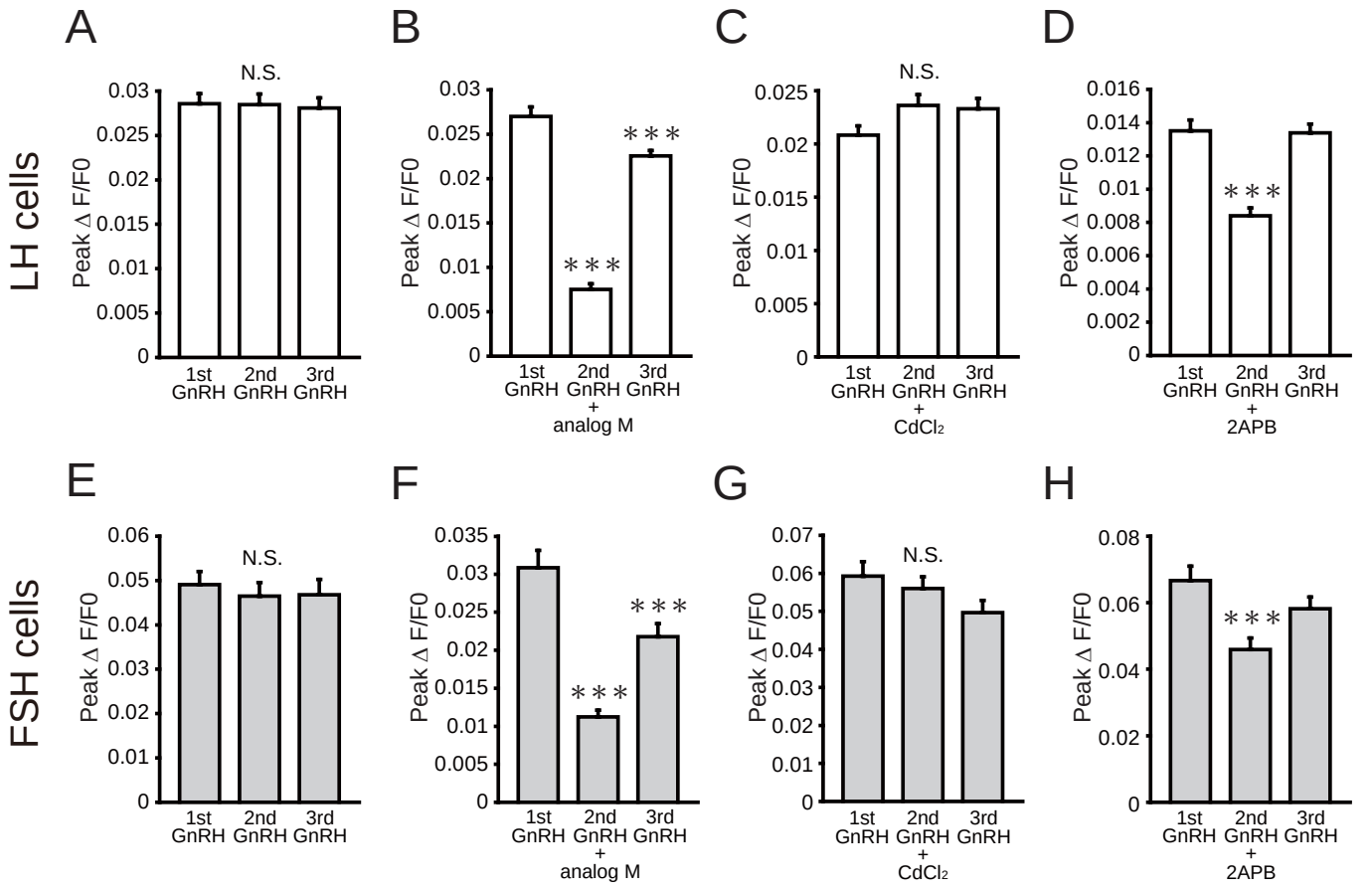
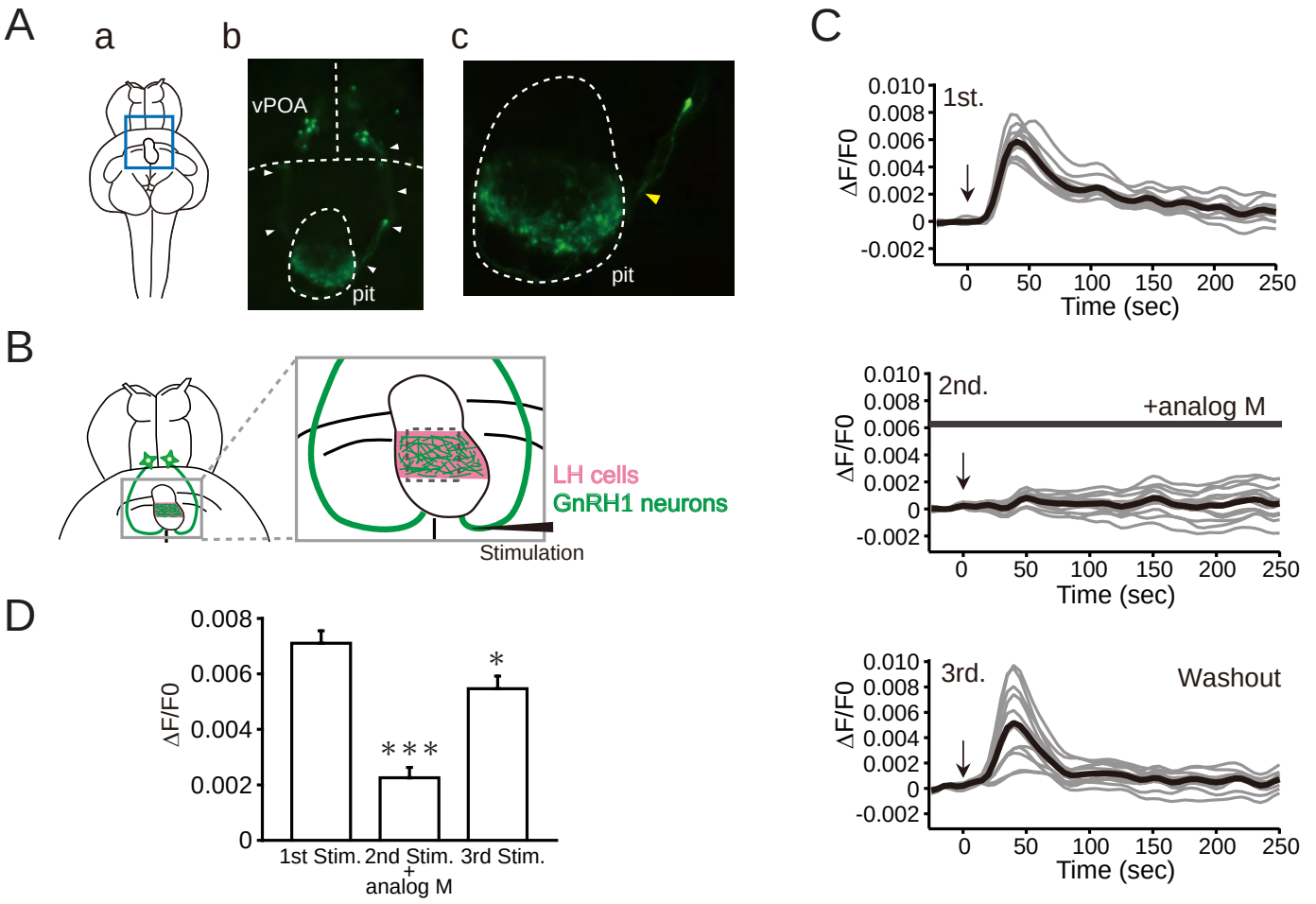
0
0.08
Time (sec)
B
E
Cell No.
C
F
% Peak

# Figure 2-5


A
B
C
D
E
F
G
H
LH cells
FSH cells
Peak Δ F/F0
N.S.
***
1st GnRH
2nd GnRH
3rd GnRH
2nd GnRH + analog M
3rd GnRH + analog M
2nd GnRH + CdCl2
3rd GnRH + CdCl2
2nd GnRH + 2APB
3rd GnRH + 2APB

# Figure 2-6


A
a
b
c
vPOA
pit
pit
B
LH cells
GnRH1 neurons
Stimulation
C
1st.
2nd.
+analog M
3rd.
Washout
ΔF/F0
0.010
0.008
0.006
0.004
0.002
0
-0.002
0
50
100
150
200
250
Time (sec)
D
ΔF/F0
0.008
0.006
0.004
0.002
0
* * *
*
1st Stim.
2nd Stim. + analog M
3rd Stim.

# Chapter 3

## Identification of the Regulator of GnRH1 Neurons in Some Teleosts

# General Discussion

The present thesis aimed at analyzing the basic principles of the HPG axis regulation mechanisms in vertebrates by using teleost fishes, medaka and goldfish, as models, with special reference to the hypophysiotropic GnRH1 neurons.

In Chapter 1, I showed the time-of-day changes in the GnRH1 neuronal activities and the gonadotropin mRNA expressions in the pituitary to establish the bases for studying the HPG axis regulation mechanisms in medaka. I recorded the neuronal activity of pituitary-projecting vPOA GnRH1 neurons and found time-of-day dependent changes in neuronal activities; the overall firing activity in the afternoon was higher than in the morning, and they peaked before the estimated LH surge timing. This increase of neuronal activity may initiate the LH surge and subsequently trigger ovulation. This is the first report in vertebrates showing the time-of-day dependent changes in the electrical activity of GnRH1 neurons in the neural circuit-intact brain without manipulations of the sex steroid milieu by taking advantage of the characteristics of a daily ovulating small teleost, medaka.

Concerning the dynamic changes in the spontaneous activity of GnRH1 neurons, there have been some reports on the occurrence of time-of-day dependent changes in GnRH1 neuronal activity using an ovariectomized and estradiol-treated (OVX+E) mice model (48). In the intact mice, serum 17β-estradiol (E2) concentration fluctuates along the estrous cycle and shows LH surge and ovulation every four or five days, whereas the OVX+E mice whose E2 level is kept at high concentration are supposed to mimic the proestrous phase of estrous cycle and show LH surge-like serum LH rise in every afternoon (48). In females, the ovarian steroid hormones exert both negative and positive feedback to modulate GnRH1 neuron functions and pituitary responsiveness to GnRH peptide. At proestrous phase, estradiol feedback switches from negative to positive action, causing the GnRH surge (15, 17, 18). In

such daily surge model experiments, the neuronal activities were measured only during artificially mimicked proestrous-like phase, and there have been no report concerning other phases of the estrous cycle. However, the spontaneous firing rate of GnRH1 neurons was low in the morning (AM) and high in the afternoon (PM) in the OVX+E mice, and no such difference was apparent in the OVX mice. In addition, the neuronal activity of the GnRH1 neurons in the OVX+E mice is lower than that of the OVX mice in AM and higher in PM, suggesting the existence of estrogen positive and negative feedback on GnRH1 neuronal activities; E2 facilitates the GnRH neuronal activity in AM and suppresses that in PM (48). By using a quantitative expression analysis in the OVX and sex steroid-treated medaka, the sex steroids have been shown to have a positive feedback effect on LH concerning mRNA expression in the medaka pituitary (30), which is completely opposite to the mammalian gonadotropin response to sex steroids (reviewed in (134)), and negative feedback regulation on LH have not been demonstrated in teleosts. In my experiments, the firing frequency of GnRH1 neurons tended to be the lowest prior to the estimated time of LH surge in medaka (around 16:00 to 17:00) and the highest after that (Fig. 1-2 E and F, group d and e, not statically significant). In medaka, plasma E2 concentration fluctuates in a day and is lowest at the latter of the lighting period (comparable to around 16:00 in my experiments) (135). This result may imply the existence of both negative and positive feedback mechanisms by sex steroid on GnRH1 neuronal activities also in medaka.

The time-of-day dependent changes in GnRH1 neuronal activities in both our study and the previous studies using mice suggest that some kinds of circadian neural mechanisms may be important for the regulation of GnRH neuronal activities. There are reports in rodents suggesting that signals arising from the

suprachiasmatic nucleus (SCN), which is known as the candidate master biological clock in mammalian brain, affect the hypothalamic neuronal functions such as the regulation of timing of LH surge (reviewed in (53)). However, in teleosts, the notion of master circadian clock has not yet been established. Therefore, we still do not know what the actual trigger of time-of-day changes in the activity of GnRH1 neurons in our teleost model is. Because most teleosts have rather transparent body compared with mammals, and non-mammalian vertebrates express various opsin-like photoreceptors widely outside the retina (136), certain information from light maybe transmitted to the GnRH1 neurons. Thus, the sex steroid concentration and the information from light may act as triggers in teleosts. Because the trigger of LH surge is important for the maintenance of estrous cycle, the comparison among animals of differences in the trigger of LH surge should be an interesting future topic for the elucidation of the common mechanisms of the HPG axis regulation in vertebrates.

In Chapter 1, I discussed the activities of single GnRH1 neurons. On the other hand, it has been suggested from various studies in mammals that coordination of activity of multiple GnRH neurons probably underlies two different modes of rhythmic GnRH release, GnRH pulses and surges. For example, single unit activity extracted from hypothalamic multiunit electrical recordings during LH release (MUA volleys) has indicated changes in activity of multiple neurons (137). In addition, total GnRH peptide content in GnRH neurons have been estimated to be approximately 1.0 pg/cell (138), and 30% of which likely comprises the releasable pool of GnRH peptides. The levels of GnRH in the hypophyseal portal vessels during a pulse (139, 140) (several hundred pg/ml) suggest that multiple GnRH neurons are probably involved in the release of LH. However, the mechanisms for synchronous

firing in the hypothalamic GnRH neurons are unknown, and there are quite a small number of studies that shows the coordinated activity of hypophysiotropic GnRH1 neurons (141). One of the reasons which make difficult to elucidate the coordinated activities of GnRH1 neurons is a sparse distribution of GnRH1 neurons in the brain (4). The other one may be a technical problem in that mammalian brain researchers inevitably use brain slices for physiological experiments, in which neural circuits are disrupted during slicing. In fact, some reports suggest that the GnRH1 neuronal activities and the mode and/or frequency of synaptic inputs to the GnRH1 neurons vary depending on the cutting direction of the brain slices and their thickness (50, 142). There are some implications about the interactions among individual GnRH1 neurons. From the electron microscopic studies of mice GnRH1 neurons, dendro-dendritic interactions between GnRH1 neurons are reported (143). The dendrites of the great majority of GnRH neurons (86%) form multiple close appositions with dendrites of other GnRH neurons, and puncta and zonula adherens were found connecting adjacent dendritic elements of GnRH neurons. Also, individual afferent axon terminals were found to synapse with multiple GnRH neuron dendrites in bundles. Of course, coordinated activities of hypophysiotropic GnRH1 neurons have not yet been identified in teleosts. In medaka, vPOA GnRH1 neurons directly project to the pituitary by forming distinctive thick fiber bundles (39). In those axon bundles, GnRH1 fibers are closely apposed to each other, and some kind of electrical interactions, such as electrical field effects, may be working among the axons of GnRH1 neurons in the bundles. Our whole brain preparation should prove to be a potent tool to study such synchronization mechanisms of GnRH1 neuron activities.

Next, in Chapter 2, I analyzed the effects of GnRH on the release of LH and FSH

separately. Despite the different secretory profiles of LH and FSH, many previous studies focused on LH release by GnRH, and the mechanisms of differential regulation of the release of two types of gonadotropins by GnRH are still unknown. Therefore, I generated transgenic medaka lines that express fluorescent $Ca^{2+}$ indicator proteins in either LH or FSH cells to analyze intracellular $Ca^{2+}$ dynamics separately. I performed a cell-type specific $Ca^{2+}$ imaging of LH and FSH cells and demonstrated that LH and FSH cells show different modes of $Ca^{2+}$ rise induced by GnRH. Since the $Ca^{2+}$ rise can be considered as the indicator of exocytotic release of hormones from the gonadotropes, these results indicate that the differential LH and FSH release patterns are induced by GnRH. Because of the technical limitations, I have not yet been able to measure the released hormones themselves and cannot determine the actual hormone release patterns. However, the $Ca^{2+}$ rise generally is a good indicator to know the timing of exocytosis. The previous electron microscopic study in rat gonadotropes showed that LH and FSH tend to be packaged into different kind of vesicle in identical cell, LH in small secretory granules and FSH in large secretory granules (144). In mammals, gonadotropes may control the different secretory pattern of each gonadotropin via different regulation of each kind of secretory vesicle. I also observed spontaneous $Ca^{2+}$ fluctuations in both LH and FSH cells and found that the pattern of spontaneous $Ca^{2+}$ fluctuation was different between LH and FSH cells. Spontaneous $Ca^{2+}$ fluctuations tended to be reduced under GnRH antagonist treatment but did not completely diminish in both LH and FSH cells. This suggests that LH and FSH cells receive additional inputs from unidentified non-GnRH neurons, and such inputs may be different between LH and FSH cells. In this respect, the study of gonadotropes using teleosts, in which LH and FSH cells are separated as different cell groups, should go a long way towards the

understanding of the neuroendocrine regulation mechanisms of each cell group, and we may be able to find out a novel regulatory mechanism for gonadotropin secretion by using the novel experimental model as shown here in Chapter 2.

In Chapter 3, I searched for neural inputs to the GnRH1 neurons to study the control mechanisms of GnRH1 neurons, focusing on two molecules, kisspeptin and dopamine (DA). In mammals, the kisspeptin signaling is now considered to be essential for the HPG axis regulation; kisspeptin strongly activates GnRH1 neuronal activities and induces serum LH elevation and ovulation. However, unlike in mammals, neither *in vivo* i.p. nor i.c.v. administration of kisspeptin induced plasma LH elevation and ovulation in sexually mature goldfish. In addition, kisspeptin did not change the firing frequency of GnRH1 neurons in medaka. In contrast, DA, which has been suggested to have inhibitory effects on the HPG axis in some mammals telosts, significantly inhibited the GnRH1 neuronal activities in medaka. I also found close appositions between DA neurons and GnRH1 neurons in both vPOA and the pituitary. In addition, LH and FSH cells were innervated by dopaminergic fibers.

In spite of many reports on the mammalian kisspeptin actions as a potent activator of GnRH1 neuronal activity, I could never find any positive effects of kisspeptin on the activation of the HPG axis in medaka or goldfish. Combined with the recent reports that demonstrated the absence of Gpr54 (kisspeptin receptor) on GnRH1 neurons in some teleosts (102-104), it is strongly suggested that, in teleosts, kisspeptin may not modulate GnRH1 neurons in the same manner as that of mammals; it may not even regulate the HPG axis in non-mammalian species in general. Recently, the involvement of kisspeptin systems in various physiological functions other than reproduction have been shown in teleosts. For example, in

medaka, a subpopulation of vasotocin and isotocin neurons express *gpr54-2* mRNA, and these neurons are suggested to be related to the regulation of social behaviors (103). In the European sea bass, *gpr54-2* mRNA is expressed in somatostatin neurons and NPY neurons, which are suggested to be related to the control of GH secretion and feeding, respectively (104). Interestingly, mammals and most of the non-mammalian vertebrates possess kisspeptin systems, and only avian species seem to have lost kisspeptin systems; both the genes for kisspeptin and its receptor GPR54 are lacking in birds (96). In spite of the absence of kisspeptin system, the avian species retain a normal ability of reproduction. This fact implies a possibility that kisspeptin system originally had some physiological functions other than the HPG axis regulation and have evolved and specialized as a regulator of HPG axis regulation in mammals. In this hypothesis, non-mammalian vertebrates including teleosts may have some kind of regulatory mechanisms to regulate GnRH1 neurons other than kisspeptin neurons, and those mechanisms may be truly essential for the HPG axis regulation. Until now, *Kiss1* or *Gpr54* gene knockout animals have been reported only in mammals, human (hereditary disorder) and rodents. Therefore, gene knockout studies in non-mammalian vertebrates, which have become available as TALEN or CRISPR/Cas9 technology, may provide novel insights into the understanding of the candidate original function(s) of the kisspeptin systems in the near future.

By contrast, the inhibitory effect of DA on the HPG axis studied in Chapter 3 seems to be common between mammals and teleosts. There are several reports that measured serum LH and GnRH concentrations after treating animals with DA agonist and antagonist in both mammals and teleosts (60, 110-112, 125). However, the direct inhibitory effect of DA on the GnRH1 neurons were shown for the first

time by electrophysiological study in mice, quite recently (114) and needs further detailed analysis in future. In Chapter 3, I demonstrated that DA inhibits the activities of GnRH1 neurons for the first time in teleosts, and this inhibitory effect appears to be similar to that shown in mice (114). In fact, the inhibitory effect of DA on the GnRH1 neuronal activities was significant but transient. Although such transient inhibitions on GnRH1 neuronal activities themselves may not have a potent influence on the total GnRH release at the pituitary, DA treatment significantly inhibited GnRH release from the hypothalamic brain explants (60, 125). Dopaminergic inhibition may have additional effects other than mere inhibition of GnRH1 neuronal activities, e.g., inhibition of transcription of GnRH or modulation of the control neural circuit for GnRH1 neurons. I also showed a possible direct inhibition on LH and FSH cells. The direct dopaminergic inhibitory effects on LH release or synthesis have been suggested in both mammals (145-147) and teleosts (61, 115, 148, 149). Overall, the dopaminergic inhibitions on both GnRH1 neurons and LH cells (gonadotropes in mammal) are possibly common to many vertebrates. As the number of studies on the dopamine effects on FSH cells is rather small, elucidation of the differential regulation of DA on gonadotropes should be interesting.

In summary, the present thesis revealed several novel aspects of the HPG axis regulation mechanisms in teleost fishes with special focus on the hypophysiotropic GnRH1 neurons and the pituitary, by taking advantage of medaka as a model system for the study of such mechanisms in vertebrates. The HPG axis regulation mechanisms in vertebrates have been studied for a long time; more than 40 years have passed after the discovery of GnRH, and considerable amount of knowledge on the individual components of the HPG axis regulation has been accumulated until

now. However, we are still far from the comprehensive understanding of the entire neural circuitry for the HPG axis regulation mechanisms, because the cellular level analysis of the neural circuitry for the HPG axis regulation under nearly intact physiological conditions has been fairly difficult. I believe that my medaka model will go a long way towards the elucidation of the long-standing problems in the neuroendocrine study of reproduction and provide profound insights into the truly essential common regulatory mechanisms of vertebrate reproduction through the comparison of studies in various mammalian as well as non-mammalian vertebrate species.

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