学位論文（要約）

Physiological studies on peptidergic neurons that act as sensors for physiological state of the animal

（動物の生理状態を感知するペプチドニューロンの生理学的研究）

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List of abbreviations

ACSF	artificial cerebrospinal fluid
AgRP	agouti-related peptide
AMPK	AMP-activated protein kinase
ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
BOSS	bride of sevenless
CV	coefficient of variation
EGFP	enhanced green fluorescent protein
FSH	follicle-stimulating hormone
GnRH	gonadotropin releasing hormone
GSI	gonad somatic index
HE	hematoxylin and eosin
HPG axis	hypothalamic-pituitary-gonadal axis
I_{CaT}	T-type calcium currents
I_h	hyperpolarization-activated currents
I_{NaP}	persistent sodium currents
I_{SK}	small conductance calcium-activated potassium currents
K_{ATP} channels	ATP-sensitive potassium channels
Kiss	Kisspeptin
LH	luteinizing hormone
MS-222	tricaine methanesulfonate
NPY	neuropeptide Y

NRL	nucleus recessus lateralis
NVT	nucleus ventralis tuberis
OVX	ovariectomy
OVX+E	ovariectomy+estrogen-treatment
PeN	anterior periventricular nucleus
POA	preoptic area
POMC	pro-opiomelanocortin
RMP	resting membrane potential
RP3V	rostral periventricular area of the third ventricle
SD	standard deviation

Abstract

Animals sense changes in physiological states and adaptively modulate their behaviors, endocrine functions, and homeostatic responses. Although such modulations are well known and thought to be critical for life, the central mechanisms of the physiological state-dependent modulation remain elusive. It remains to be clarified how the central nervous system, which is a main regulator of various endocrine functions and behaviors, sense the physiological states. In the present thesis, I analyzed such central mechanisms by focusing on reproduction, which is essential for all animals and known to be regulated by various physiological states such as serum steroid hormone levels, nutrition, etc. Hypothalamic peptidergic neurons, gonadotropin releasing hormone (GnRH) neurons have been known to play essential roles in the regulation of reproduction throughout vertebrates. GnRH peptide, which is produced in the GnRH neurons and released to the pituitary, in turn, induces pituitary luteinizing hormone (LH) release from the LH cells and then ovulation. Recent studies in mammals further suggest that hypothalamic kisspeptin neurons are essential for the regulation of reproduction by directly activating GnRH neurons. Based on these backgrounds, I hypothesized that these hypothalamic peptidergic neurons sense the physiological states and properly modulate reproduction. Here, I performed physiological analyses on the relationship between the neuronal activities of peptidergic neurons and the physiological states, breeding state and nutritional state, which strongly affect reproduction, by using a teleost medaka, which has many experimental advantages for the present study.

In Chapter 1, I first examined whether steroid sensitive kisspeptin (Kiss1) neurons change their spontaneous neuronal activities according to the breeding state by using the transgenic line of medaka, which expresses enhanced green fluorescent protein (EGFP) specifically in Kiss1 neurons. Electrophysiological analyses demonstrated that Kiss1 neurons of medaka under a breeding condition show a variety of firing patterns: bursting, regular, irregular, and silent. On the

other hand, under a non-breeding condition, most of Kiss1 neurons showed silent, and much lower neuronal activities than those under a breeding condition. Since the sex steroid level should be altered in accordance with the breeding state, I finally analyzed the effects of a sex steroid hormone, estrogen, on the neuronal activities of Kiss1 neurons. The spontaneous neuronal activity of Kiss1 neurons was changed in accordance with the estrogen level, which reflects the breeding state. These results suggest that Kiss1 neurons drastically change the spontaneous neuronal activities according to the breeding state, which may be induced by changes in the sex steroid level.

In Chapter 2, I analyzed the regulation of reproduction in accordance with the nutritional state. From the comparison of reproductive behaviors under normal and fasted conditions, it was shown in female medaka that malnutritional state by fasting strongly suppressed spawning. On the other hand, in males, long-term fasting had no significant effect on fertility and courtship behavior. Quite recently, a growing body of evidence suggests that kisspeptin neurons are not essential for reproduction in non-mammalian species, in contrast to mammalian species. As the key factor for the nutritional state-dependent regulation of reproduction, I focused on the hypothalamic GnRH (GnRH1) neurons in medaka. Concentration of blood glucose, which is the main nutrient for neurons, was drastically reduced under the fasting condition. Because a glucose-dependent regulation of neuronal activities have been reported in several types of mammalian neurons, I analyzed the effect of fasting-induced low glucose on the spontaneous firing activity of the GnRH1 neurons. Low glucose induced by fasting suppressed the spontaneous neuronal activities of GnRH1 neurons only in female medaka through the activation of ATP-sensitive potassium channels and AMP-activated protein kinase pathway. These results suggest that hypothalamic GnRH neurons show female specific-glucose sensitivity in the neuronal activity, which may mediate sexually dimorphic nutritional state-dependent regulation of reproduction at least in

medaka.

As described above, I demonstrated that peptidergic neurons, Kiss1 and GnRH1 neurons, sense the physiological states through modulation of the spontaneous neuronal activities. In conclusion, the present thesis has clearly shown some of the neural mechanisms for adaptive regulation of reproduction and homeostasis according to the physiological states.

General Introduction

Physiological states of the animals are flexibly modulated by external environment(s) such as day length, temperature, and feeding condition. In accordance with changes in the physiological states, animals show adaptive behaviors, homeostatic responses, and endocrine regulation. The central nervous system is suggested to sense such changes by receiving biological signals via general circulation (e.g. hormonal levels, metabolic status) and induce adaptive behaviors/homeostatic responses. Although we generally assume such processes, the neural mechanisms underlying the physiological state-dependent regulation remain unclear. In the present thesis, I focused on "reproduction" to elucidate the neural mechanisms that contribute to animal adaptation to the changing environment. There is no species that could exist without reproduction. Reproductive functions and behaviors are modulated by various physiological states, such as breeding state, which is regulated by seasonality, nutritional state, and stress (1-6). As reproduction cannot occur without an orchestrated regulation of these conditions, it is assumed that reproduction is one of the most suitable models for the experimental analysis of the physiological state-dependent regulation.

Among various physiological states related to reproduction, I focused on two physiological states: the breeding state and the nutritional state. These states are generally known to strongly affect the reproductive functions and behaviors. However, the neural mechanisms of modulation of reproduction dependent on these states are poorly understood. This is probably because of the fact that most physiological studies in the field of reproduction have used mammals, especially rodents, as experimental models. The rodents have many advantages for the physiological analysis but are not suitable for the studies on the breeding state and the nutritional state from the following reasons. 1) The rodents bred in the laboratory conditions have lost seasonality of reproduction. 2) The experimental manipulation of nutritional status is rather difficult due to their high basal metabolism, because they are endothermic animals.

Here, I chose medaka (*Oryzias latipes*) for my thesis study. Medaka is a seasonal breeder, and

the breeding state can be manipulated solely by a day length or temperature (= season). In addition, the basal metabolism of medaka is low, which enables the long-term regulation of the nutritional state. Therefore, medaka is a good model for the analysis of the relationship between physiological states and reproduction. Recently, various transgenic medaka lines, whose specific type of neurons express fluorescent proteins (e.g. GFP), have been generated and applied for physiological analyses (7-11). By taking advantage of this, I analyzed the neural mechanisms of regulation of reproduction according to the breeding/nutritional states.

Because the external environment changes gradually, the adaptive modulation of behaviors/ endocrine functions is considered not to be acute but to be chronic. Thus, the present thesis focused on peptidergic neurons as a key factor in such chronic modulation. The peptidergic neurons play an essential role in the modulation of various neural and endocrine systems by the release of neuropeptides. As the characteristic of peptidergic neurons, various peptidergic neurons show spontaneous neuronal activities (7,8,12-19) that are dependent on intrinsic neuronal properties such as ion channels. Although the role of spontaneous firing activity is not completely understood, the release of neuropeptide may be related to this activity. Thus, I hypothesized that the spontaneous firing activities in peptidergic neurons are continuously modulated in accordance with the physiological states, which contributes to the chronic physiological state-dependent modulation by the release of neuropeptide.

In vertebrates, the hypothalamic-pituitary-gonadal (HPG) axis is essential for the regulation of reproduction. Hypothalamic peptidergic neurons, gonadotropin-releasing hormone (GnRH) neurons, are conserved throughout vertebrates (20-22) and known to play an important role in the HPG axis regulation. During the past few decades, another hypothalamic peptidergic neuron, the kisspeptin neuron, has also been reported to be necessary for the regulation of reproduction in mammals. In mammals, the kisspeptin neurons receive the sex-steroids from the gonads and promotes fertility by directly activating the hypothalamic GnRH neurons (23,24). Based on these

studies, I assumed that the two peptidergic neurons, GnRH neurons and kisspeptin neurons, mediate the regulation of reproduction depending on the physiological states.

In the present thesis, I first analyzed the sex steroid regulation of the spontaneous neuronal activity of the kisspeptin neurons, because they have been suggested to regulate reproduction by activating the GnRH neurons in mammals (Chapter 1). However, growing body of recent evidence suggests that the kisspeptin neurons are not directly involved in the central regulation of reproduction at least in some teleosts including medaka (25-27). Therefore, I next focused on the hypothalamic GnRH (GnRH1) neurons and examined whether the spontaneous neuronal activity of the GnRH1 neuron is modulated by another physiological state, nutritional state (Chapter 2).

Chapter 1

Kiss1 neurons drastically change their spontaneous firing activity in accordance with the breeding state: insights from a seasonal breeder

Abstract

Kisspeptin (Kiss) neurons show drastic changes in kisspeptin expression in response to the serum sex steroid concentration, which changes according to breeding condition in various vertebrate species. Thus, according to the breeding state, kisspeptin neurons are suggested to modulate various neuronal activities, including the regulation of GnRH neurons in mammals. However, despite their breeding state-dependent regulation, there is no physiological analysis of kisspeptin neurons in seasonal breeders. Here I performed electrophysiological analyses of steroid-sensitive Kiss1 neurons in the nucleus ventralis tuberis (NVT) by using *kiss1*: EGFP transgenic line of a seasonal breeder, medaka. By using a whole-brain *in vitro* preparation in which most synaptic connections are intact, I revealed that they show various firing patterns, including bursting. Furthermore, I found that their firings are regulated by the resting membrane potential. However, bursting was not induced from the other firing patterns with a current injection, suggesting that it requires some chronic modulations of intrinsic properties such as ion channel expression. Finally, I found that NVT Kiss1 neurons drastically change their spontaneous neuronal activities according to the breeding state and the estradiol levels. Taken together with the previous reports, I here conclude that the breeding condition drastically alters the Kiss1 neuron activities in both spontaneous firing activities and gene expression, the former of which is strongly related to Kiss1 release, and the Kiss1 peptides regulate the activities of various neural circuits.

Introduction

The kisspeptin neuronal system is now considered an essential component for reproduction in mammals because knockout or mutation of the ligand gene *Kiss1* or the receptor gene *Gpr54* results in reproductive dysfunction in mice and humans (28-32). This action is suggested to be mediated by GnRH neurons, as evidenced by electrophysiological experiments in mice (33-37). In addition to *kiss1, kiss2* was found in many vertebrate species, including some mammals, and these paralogous genes (*kiss1* and *kiss2*) were duplicated before the emergence of lamprey, thus the

vertebrate lineage originally had both *kiss1* and *kiss2*, while some groups of animals lost *kiss1* and/or *kiss2* (38-43).

As the unique characteristic of theses kisspeptins among vertebrates, the kisspeptin gene expression changes according to the serum sex steroid level (44). Since the sex steroid level is strongly correlated to breeding state, kisspeptin neurons may sense the breeding condition through the change in the sex steroid level. Recently, in addition to their regulation of GnRH neurons in mammals, it has been suggested that kisspeptin is involved in the regulation of oxytocin and vasopressin neurons in mammals (45,46) , isotocin and vasotocin (orthologous to mammalian oxytocin and vasopressin, respectively) in medaka (25), which are supposedly involved in the homeostatic regulation and social behaviors. Therefore, kisspeptin neurons may relay the breeding condition to breeding season-specific homeostatic responses and behaviors among vertebrate species.

However, in spite of such importance of kisspeptin neurons in the breeding state-dependent modulation, the physiological properties of kisspeptin neurons are completely unknown in seasonal breeders. Among seasonal breeding animals, I chose medaka because seasonal breeding is determined solely by day-length in medaka. In addition, they have the unique advantage of being amenable to transgenic techniques and physiological recordings without interrupting major synaptic connections using a whole-brain *in vitro* preparation. In medaka, Kiss1 neurons in the hypothalamic nucleus and nucleus ventralis tuberis (NVT), but not Kiss2 neurons in nucleus recessus lateralis (NRL), are steroid sensitive, which suggests the NVT Kiss1 neurons are responsible for relaying the peripheral breeding state and altering the central regulation of hormonal and behavioral regulation in medaka (41,42).

In this chapter, I analyzed electrophysiological properties of steroid sensitive Kiss1 neurons in medaka by using *kiss1*: enhanced green fluorescent protein (EGFP) transgenic line (47). In addition, I analyzed not only the basic spontaneous firing activity but also the difference in spontaneous firing activity between breeding and non-breeding conditions.

Material and Methods

Animals

kiss1: EGFP transgenic medaka (*Oryzias latipes*), whose Kiss1 neurons specifically express EGFP (47), were maintained under a 14 h light/10 h dark photoperiod at a temperature of 27 °C. The fish were fed twice daily with live brine shrimp and flake food. The animals were maintained and used in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) and the guidelines of the University of Tokyo for the Use and Care of Experimental Animals.

Electrophysiology

Using the sexually mature *kiss1*: EGFP transgenic medaka, I performed whole-cell patch-clamp recordings from genetically GFP-labeled Kiss1 neurons in the daytime (11:00~20:00). Adult male and female transgenic medaka were anesthetized by immersion in 0.02% tricaine methanesulfonate (MS-222) and decapitated. The whole brain was then rapidly removed and placed in a hand-made chamber filled with an artificial cerebrospinal fluid (ACSF) containing 134 mM NaCl, 2.9 mM KCl, 1.2 mM $MgCl_2$, 15 mM glucose, 10 mM HEPES, and 2.1 mM $CaCl_2$, (pH 7.4, adjusted with NaOH). Whole-brain *in vitro* preparations for the patch-clamp recordings were prepared by using fine forceps to carefully peel off the meninges covering the hypothalamus. Under an upright fluorescent microscope with infrared (IR)-differential interference contrast (DIC) optics (Eclipse E-600FN; Nikon, Tokyo, Japan) and an IR CCD camera (C3077-78, Hamamatsu photonics, Hamamatsu, Japan), Kiss1-GFP neurons were easily identified by their florescence. The patch pipettes were made from borosilicate glass capillaries of 1.5-mm outer diameter (GD-1.5; Narishige, Tokyo, Japan). They were pulled using a Flaming-Brown micropipette puller (P-97; Sutter Instruments Co., Novato, CA). The tip resistance of patch pipettes in the ACSF was approximately 15–30 MΩ. Targeted whole-cell patch recordings were performed with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA),

digitized (10 kHz), and stored on a computer using Digidata 1322A and pCLAMP 9.2 software (Molecular Devices, Sunnyvale, CA). Pipette solution containing 112.5 mM K^+-gluconate, 4.0 mM NaCl, 17.5 mM KCl, 1.0 mM $MgCl_2$, 1 mM EGTA, 10 mM sucrose, 10 mM HEPES, and 0.5 mM $CaCl_2$, (pH 7.2, adjusted with KOH). After formation of the gigaohm seal, zap was applied to form the whole-cell recording mode. Various spontaneous activities were recorded in current clamp mode. For the whole-cell configuration, access resistance was less than 300 MΩ. The liquid junction potential of 13.8 mV was corrected off-line for all analyses.

Comparison of the fluorescence and firing activities of GFP-labeled NVT Kiss1 neurons in the breeding and the non-breeding conditions

To compare the GFP-fluorescence and firing activities between the breeding and the non-breeding conditions, pairs of male and female transgenic medaka were maintained under a breeding (14-h light and 10-h dark) and a non-breeding condition (10-h light and 14-h dark) at 27 °C for 2 to 5 weeks, as they are long-day breeders. Fluorescent photomicrographs of GFP-labeled NVT Kiss1 neurons in the breeding and the non-breeding conditions were taken at same exposure time (1.1 s), gain, intensity. Additionally, at the same time on the same day, these fluorescent photomicrographs were taken under the same condition. Brightness and contrast were adjusted with all the same value for every picture in each experiment using Adobe Photoshop CS6**.**

Ovariectomy (OVX) and estrogen replacement

For the OVX and estrogen replacement experiments, I used female *kiss1*: EGFP transgenic medaka, which had been kept in a breeding condition and were sexually mature. After female medaka was anesthetized by immersion in 0.02% MS-222, a 2- to 3-mm incision was made along the abdomen of the fish, and the whole ovary was removed (OVX). After the OVX, the incision was sutured with a nylon thread. For sham-operated medaka (Sham), the fish underwent all the

aforementioned operations except for the removal of the ovaries. For ovariectomy and estrogen replacement medaka (OVX + E), 1×1×1 mm silicon cube (KE-106 mixed with catalyst, CAT-R; Shin-Etsu Chemical, Tokyo, Japan) containing approximately 0.04 mg 17β -estradiol (Sigma, St. Louis, MO) was implanted after the removal of the ovary (48). The operated fish were maintained under a 14 h light/10 h dark photoperiod at a temperature of 27 °C without feeding. After keeping the operated fish for three days, I used the fish for electrophysiological analyses.

Data analysis and classification of firing pattern

All values are shown as the mean ± SEM. For calculation of the resting membrane potential (RMP), the RMPs at 1, 2, 3, 4, 5, and 6 min after stable whole-cell patch recording started were measured and averaged. For the classification of the firing patterns in whole-cell patch-clamp recordings, instantaneous spike frequency (reciprocal to the interval between spikes, Hz) and inter-spike interval were calculated from the recordings during the time between 1 and 6 min after the start of the stable recordings. I classified the various firings of Kiss1 neurons into four different firing patterns. The classification of firing patterns was defined as follows. Bursting: the trace that includes three consecutive action potentials at intervals less than 500 msec. Regular firing: the coefficient of variation (CV) of the inter-spike interval was less than 0.65. The CV is defined as the ratio of the standard deviation (SD) to the mean inter-spike interval. Silent: 1-min bin showed one action potential or less. Irregular firing: recordings that did not match any of the three definitions above. In silent neurons that exhibited no spontaneous activity, I confirmed recording success by inducing several firings via current injection (1~20 pA). Statistical analyses were performed with Kyplot5 (Kyence, Tokyo, Japan) and Igor Pro 6 (WaveMetrics Inc., Lake Oswego, OR). I used the Mann-Whitney *U* test for the statistical comparisons between two groups. The comparisons between control and multiple groups were performed by the Steel test. For the comparison between all groups, the statistical analyses were performed by the Steel-Dwass test. The statistical analyses for the comparisons in the proportions of four firing patterns, χ^2 test was

used. Significant difference was defined as $P < 0.05$.

Results

NVT Kiss1:GFP neurons exhibit a variety of firing patterns

In medaka, among Kiss1 neuronal populations in the brain, the NVT Kiss1 neurons show significant steroid sensitivities (49). Therefore, I focused the electrical recordings on the NVT Kiss1 neurons. First, I recorded spontaneous firing activities from mature male and female medaka under the breeding condition. By whole-cell patch-clamp recordings, I found that NVT Kiss1 neurons from males exhibited an average RMP of −56.7 ± 1.5 mV (range −41.3 mV to −74.0 mV, n = 29 neurons from 24 fish). Similar to those in males, the average RMP of NVT Kiss1 neurons from mature females was −59.7 ± 1.9 mV (range −41.4 mV to −86.6 mV, n = 29 neurons from 23 fish). Moreover, in both sexes, NVT Kiss1 neurons showed a similar mean instantaneous spike frequency (male: 0.6 ± 0.1 Hz, n = 29; female: 0.9 ± 0.2 Hz, n = 29).

Targeted recordings from NVT Kiss1 neurons in males and females revealed that Kiss1 neurons show various firing patterns: bursting, irregular, regular, and silent (Fig. 1-1A). The firing patterns of the recorded neurons were as follows: bursting, three neurons (10%); regular, six neurons (21%); irregular, fourteen neurons (48%); and silent, six neurons (21%) in males; and bursting, five neurons (17%); regular, six neurons (21%); irregular, twelve neurons (41%); and silent, six neurons (21%) in females (male: n = 29, female: n = 29; Fig. 1-1B and Table 1). There were no significant differences in the relative proportion of firing patterns between males and females (Fig. 1-1B; P =0.88; χ^2 test). I also analyzed spontaneous firing activities of NVT Kiss1 neurons by on-cell loose-patch recordings, in which the intracellular fluid can be kept in a physiological condition. Under on-cell loose-patch recordings, NVT Kiss1 neurons from male and female medaka also showed a similar firing pattern to whole-cell recordings: bursting, 15%; regular, 8%; irregular, 46%; and silent, 31% (n = 13, Supplemental Fig.1-1).

RMP determines the firing patterns of NVT Kiss1 neurons except bursting

The whole-cell and on-cell loose-patch recordings of NVT Kiss1 neurons revealed that NVT Kiss1 neurons show various firing patterns. Interestingly, the average RMP of each firing pattern from both male and female medaka was different; the average RMP of neurons showing bursting was −55.6 ± 1.8 mV (range −48.2 mV to −64.4 mV, n = 8), that of regular firing was −53.7 ± 2.5 mV (range −41.3 mV to −73.1 mV, n = 12), that of irregular firing was −55.2 ± 1.2 mV (range −41.4 mV to −69.6 mV, n = 26), and that of silent was −70.7 ± 2.3 mV (range −59.4 mV to −86.6 mV, n = 12). In particular, the average RMP of the silent neurons was significantly more negative than the neurons with other firing patterns (Fig. 1-2A; $P < 0.01$ silent vs bursting; $P < 0.001$ silent vs regular, and silent vs irregular; Steel test). Some cells, such as thalamocortical neurons and globus pallidus neurons, are reported to show changes in firing patterns in accordance with the RMP (50-52). Therefore, I examined the firing pattern modulation by RMP in NVT Kiss1 neurons. I demonstrated that silent Kiss1 neurons shifted to irregular and/or regular firing by positive current injections (Fig. 1-2B). Moreover, irregular or regular Kiss1 neurons also altered their firing pattern to silent, irregular, or regular by continuous positive or negative current injections (Fig. 1-2C). These results suggest that the RMP plays an important role in the determination of the firing pattern in NVT Kiss1 neurons. However, interestingly, NVT Kiss1 neurons did not shift to the bursting from silent, irregular, or regular firing by continuous current injections.

NVT Kiss1 neurons in the non-breeding condition show lower firing activities than those in the breeding condition

In medaka, the number of *kiss1*-expressing neurons in NVT decreases under the non-breeding condition (49). I examined if the spontaneous neuronal activities of NVT Kiss1 neurons also change according to the breeding state. Consistent with the previous report of *kiss1* mRNA variation (49), the comparison of the fluorescence of GFP-labeled NVT neurons under the same observation conditions showed that the fluorescence in the non-breeding condition was obviously

weaker than that in the breeding condition (Fig. 1-3A,B). This result suggests that the 5' flanking region (~3 kb) of the *kiss1* gene used in this transgenic medaka (47) contains enhancers/promoters that are essential for estrogen-dependent expressional regulation.

I compared the spontaneous firing activity between the breeding and non-breeding conditions using whole-cell patch-clamp recordings. In non-breeding, most of the NVT Kiss1 neurons from males and females showed a silent pattern (male: 78%, female: 67%), and bursting/regular neurons were not found (Fig. 1-3C and Table 1). Similar to the result in the breeding condition, the average RMP of the silent neurons from both male and female was significantly more negative than that of the neurons with irregular patterns [silent: −62.9 ± 1.4 mV (n = 19), irregular: −57.3 ± 2.7 mV (n = 8); $P < 0.05$ silent vs irregular; Mann-Whitney *U* test]. The relative proportion of firing patterns differed significantly between the breeding conditions and the non-breeding conditions (Fig. 1-3C; male: $P < 0.05$ breeding vs non-breeding; female: $P < 0.01$ breeding vs non-breeding; χ^2 test). Kiss1 neurons in the non-breeding condition from male and female medaka showed a significantly lower mean instantaneous spike frequency than that in the breeding condition (Fig. 1-3D; breeding: 0.8 ± 0.1 Hz, n = 58 cells, non-breeding: 0.1 ± 0.1 Hz, n = 27 cells, $P < 0.001$ breeding vs non-breeding; Mann-Whitney *U* test). The average RMP of NVT Kiss1 neurons in the non-breeding condition from both male and female was −61.3 ± 1.3 mV (range −46.3 mV to −73.4 mV, n = 27), which was significantly hyperpolarized compared to −58.2 ± 1.2 mV (n = 58) in the breeding condition (Fig. 1-3E; $P < 0.05$ breeding vs non-breeding; Mann-Whitney *U* test). These results suggest that NVT Kiss1 neurons exhibit low spontaneous neuronal activities under non-breeding conditions.

Changes in estrogen levels alter neuronal activities of NVT Kiss1 neurons

In mammals, the previous report suggested that estradiol regulates electrophysiological properties of Kiss1 neurons in mice , besides *kiss1* gene expression (53). For examining the effects of estrogen levels on spontaneous neuronal activities of NVT Kiss1 neurons, I analyzed their

spontaneous neuronal activities among the groups of sham operated (Sham), OVX, and OVX + estrogen-treated (OVX + E) female fish. By comparing the fluorescence between OVX and OVX + E groups, I found that the EGFP fluorescence in the OVX medaka was obviously weaker than that in OVX + E medaka (data not shown). Most of the NVT Kiss1 neurons in the OVX group showed a silent firing pattern (67%), the rest of them showed irregular firing, and none of them showed bursting or regular firing patterns, which was similar to the results for non-breeding conditioned fish (Fig. 1-4A and Table 1, n = 15 cells). On the other hand, NVT Kiss1 neurons in Sham and OVX + E fish showed various firing patterns including bursting (Fig. 1-4A and Table 1, Sham: n = 12, OVX + E: n = 14). Additionally, the percentages of silent neurons in Sham and OVX + E groups were lower than that in OVX group (Fig. 1-4A and Table 1, Sham: 50%, OVX: 67%, OVX + E: 29%). These results suggest that the proportion of firing patterns in NVT Kiss1 neurons varies according to the estrogen levels. Furthermore, mean instantaneous spike frequency of Kiss1 neurons in OVX fish is marginally significantly lower than that in OVX + E group and is rather lower than that in Sham group (Fig. 1-4B; Sham: 1.0 ± 0.5 Hz, OVX: 0.1 ± 0.1 Hz, OVX + E: 0.5 ± 0.1 Hz, $P < 0.1$ OVX vs OVX + E; Steel-Dwass test). The average RMP of NVT Kiss1 neurons in OVX fish tended to be hyperpolarized compared with that in the OVX + E fish but was similar to that in Sham (Fig. 1-4C; Sham: −68.0 ± 3.5 mV, OVX: −69.1 ± 2.7 mV, OVX + E: −59.7 ± 3.3 mV, $P < 0.1$ OVX vs OVX + E; Steel-Dwass test). These results suggest that NVT Kiss1 neurons change their spontaneous neuronal activities in accordance with the estrogen levels, although estrogen may not be the only factor that determines their firing rates.

Discussion

In this chapter, using *kiss1*: EGFP transgenic line of medaka, I performed electrophysiological experiments on Kiss1: EGFP neurons for the first time in seasonal breeders. From the present electrophysiological analysis and the previous anatomical evidence (42,49), I suggest that the breeding conditions affect both spontaneous neuronal activity and expression level of *kiss1* gene

in Kiss1 neurons. This dual-level regulation by breeding state may switch the breeding condition-specific homeostatic changes and behaviors that have been suggested by the previous experimental morphological studies.

NVT Kiss1 neurons exhibit various firing patterns

The medaka NVT Kiss1 neuron is assumed to be a good model for studying the significance and mechanisms of steroid sensitivity, which is conserved among vertebrate kisspeptin neurons. In the present study, I took advantage of the medaka whole-brain *in vitro* preparation in that one can make recordings without disrupting synaptic connections (54). It was shown that NVT Kiss1 neurons from both sexes exhibited a variety of firing patterns: bursting, irregular, regular, and silent. The characteristic firing patterns of the NVT Kiss1 neurons are similar to those in the mammalian rostral periventricular area of the third ventricle (RP3V) and arcuate nucleus (ARC) (14,55). In mammals, Kiss1 neurons in the RP3V, as well as those in the ARC show various firing patterns depending on the stage of estrous cycles (56). Although it is difficult to compare the firing activities among different animal species in different recording conditions, it is noteworthy that virtually all reports of Kiss1 neuron firing activities so far indicate such non-homogenous firing activities. Actually, medaka NVT Kiss1 neurons in any condition contained various firing patterns, even in the same animal. In contrast to this variation among individual cells, it was demonstrated that the average instantaneous spike frequency was significantly lower in the non-breeding condition, suggesting that the total release of Kiss1 from all Kiss1 neurons is precisely regulated as a population in accordance with their breeding state.

Firing patterns of NVT Kiss1 neurons are dependent on RMP

In mice, RP3V (anteroventral periventricular and anterior periventricular nuclei; AVPV/PeN) Kiss1 neurons show different RMPs between irregular firing patterns and quiescence (53). I found that NVT Kiss1 neurons also exhibit various RMPs among the four firing patterns. In addition,

among silent, irregular, and regular patterns, each pattern was shifted into one another by continuous current injections. Interestingly, in neurons such as GnRH2 and GnRH3 neurons, the firing frequency was changed by continuous current injections without changing firing patterns (8,12), the firing patterns, namely CV of inter-spike intervals, in NVT Kiss1 neurons were drastically changed by continuous current injections (Fig. 1-2). These results strongly suggest that such firing activities of NVT Kiss1 neurons are dependent on their RMP. Silent or irregular Kiss1 neurons were shifted to regular firing by depolarizing current injections. Some voltage-dependent ion currents, such as persistent sodium currents (I_{NaP}) and T-type calcium currents (I_{CaT}) coupled to small-conductance calcium-activated potassium currents (I_{SK}), are known to contribute to the generation of pacemaker (tonic) firing (57,58). Although the present study did not analyze the intrinsic ion channels of NVT Kiss1 neurons, depolarizing current injections may activate these voltage-dependent ion channel activities and shift the firing of NVT Kiss1 neurons to regular activity.

On the other hand, the bursting patterns of NVT Kiss1 neurons were not induced by the change in RMP. Here, I suggest that the bursting pattern might be induced by some chronic changes, such as the composition of expressed ion channels in the Kiss1 neurons. I_{CaT} and hyperpolarization activated currents (I_h) have been suggested to be involved in rhythmic bursting (59). Therefore, these currents might be possible candidates for generating bursting in NVT Kiss1 neurons. Voltage-clamp studies comparing bursting and tonic firing neurons may prove the generation mechanisms of the bursting of NVT Kiss1 neurons.

NVT Kiss1 neurons drastically change their neuronal activities according to the breeding conditions, which is likely to be mediated by the serum sex steroid levels

The GFP-labeled NVT neurons drastically changed their fluorescence intensity according to the breeding condition (Fig. 1-3A, B). Moreover, I also found that the intensity of GFP fluorescence in the OVX medaka was obviously weaker than that in OVX + E medaka (data not shown). These

data strongly suggest that 5' flanking region that was used to generate this transgenic line includes regulatory sequences that regulate estrogen-sensitive expression. NVT Kiss1 neurons in the non-breeding condition exhibited lower spontaneous neuronal activities than those in the breeding condition. Most of the NVT Kiss neurons showed a silent firing pattern and there were no bursting NVT Kiss1 neurons in the non-breeding condition. Furthermore, I also suggested that spontaneous firing activities of NVT Kiss1 neurons in females are likely to be regulated by estradiol. In OVX group, NVT Kiss1 neurons showed lower spontaneous neuronal activities than those in OVX+E group. On the contrary, I did not find statistical difference in the mean instantaneous spike frequencies of NVT Kiss1 neurons between Sham and OVX groups. In OVX medaka, the GFP-fluorescence of NVT Kiss1 neurons almost disappeared 4~5 days or more after the ovariectomy. Therefore, in the present study, I used the Sham/OVX/OVX+E fish 3 days after the operation, which were kept alone and fasted for recovery. Because of 3-day fasting and individual housing after the operation, ovaries in the sham-operated fish did not show any ovulated eggs, which suggests lower level of gonadal estrogen secretion. This may have caused the low level of firing in the NVT Kiss1 neurons in Sham group compared to the breeding conditioned medaka reared in pairs (Fig. 1-1, 1-4). In mice, estradiol changes the proportion of firing patterns and spontaneous IPSC amplitudes of Kiss1 neurons (53). Additionally, it has also been reported in mouse Kiss1 neurons that estradiol enhances various intrinsic ion channel currents, I_h, I_{CaT}, and I_{NaP}, which contribute to the generation of bursting and pacemaker activities (14,60,61). On the basis of these reports and the present study, it is hypothesized that the elevation of serum estrogen concentration according to the breeding state modulates the intrinsic firing properties, as well as synaptic inputs toward NVT Kiss1 neurons, which induce the bursting and regular firing of NVT Kiss1 neurons in female medaka. Unfortunately, I could not analyze the effect of sex steroids (estrogens and/or androgens) on the firing activity of Kiss1 neurons in males because of experimental difficulties to remove small testis of medaka. Future studies analyzing the effects of sex steroids on these intrinsic firing properties and the synaptic inputs of NVT Kiss1

neurons in both sexes may unveil the mechanisms of how sex steroids modulate the neuronal activities of Kiss1 neurons.

In the non-breeding condition and OVX groups, NVT Kiss1 neurons did not show bursting. The release of neuropeptide requires the high-frequency firing, such as bursting (62,63), which suggests that Kiss1 release seldom occurs in the non-breeding condition. Additionally, previous studies have indicated that the expression of the *kiss1* gene in the NVT decreases in the non-breeding condition and ovarian estrogens (42,49). Taken together, the secretion of Kiss1 peptide from the nerve terminal of NVT Kiss1 neurons may change in accordance with the breeding condition. However, it should be also noted that the release of some neuropeptides can be independent of action potential activities (64,65). Thus, future studies may be necessary that directly analyze the difference in the release of kisspeptin according to the breeding condition. Interestingly, kisspeptin receptors are reported to be expressed in isotocin, vasotocin, and somatostatin neurons (25,66), which are involved in the control of social/sexual behaviors and homeostasis. This may suggest that NVT Kiss1 neurons play important roles in the differential regulation of behaviors and homeostasis between the breeding and non-breeding seasons.

Conclusions

In summary, I revealed the spontaneous firing activity of the steroid-sensitive NVT Kiss1 neurons. Although a growing body of evidence suggests the importance of kisspeptin neurons in the regulation of vertebrate homeostasis, there had been no analysis of the physiological properties and neuronal regulation systems of kisspeptin in seasonal breeders. The physiological analyses using the whole-brain *in vitro* preparation, in which virtually no synaptic connections were disrupted, demonstrated the alteration of kisspeptin neuronal properties in accordance with the breeding state. In addition, future analyses in non-mammalian transgenic animals will reveal the differences and similarities among vertebrate species, which should highlight the most important properties of vertebrate kisspeptin neurons.

Figure Legends

Fig. 1-1. Firing activity of medaka NVT *kiss1*: EGFP neurons from the whole-brain *in vitro* preparation using whole-cell current-clamp recordings. NVT Kiss1 neurons showed an average resting membrane potential (RMP) of -56.7 ± 1.5 mV in males (n = 29), and -59.7 ± 1.9 mV in females (n = 29). A. Representative traces of spontaneous firing from NVT Kiss1 neurons. NVT Kiss1 neurons showed bursting, regular, irregular, and silent firing patterns. B. Proportions of different firing patterns in male and female.

Fig. 1-2. RMP plays an important role in determination of firing pattern in NVT Kiss1 neurons. A. Mean (±SEM) RMP of NVT Kiss1 neurons in the different firing groups. The average RMP of bursting was -55.6 ± 1.8 mV (n = 8), that of regular firing was -53.7 ± 2.5 mV (n = 12), that of irregular firing was -55.2 ± 1.2 mV (n = 26), and that of silent was -70.7 ± 2.3 mV (n = 12). The average RMP of silent neurons was significantly more negative than the other firing patterns (**: $P < 0.01$ silent vs. bursting, ***: $P < 0.001$ silent vs. regular, and silent vs. irregular; Steel test). B-C. Resting membrane potential (RMP) modulates the firing patterns of NVT Kiss1 neurons. B. A representative trace of a silent NVT Kiss1 neuron using a whole-cell current-clamp recording (left). This silent Kiss1 neuron was depolarized and shifted to the irregular firing pattern by +1.5 pA continuous current injection (CV = 0.97, middle), and to the regular firing pattern by +3.0 pA continuous current injection (CV = 0.37, right). C. A representative trace of an irregular NVT Kiss1 neuron using a whole-cell current-clamp recording (CV = 0.89, middle). This irregular Kiss1 neuron was hyperpolarized and shifted to the silent firing pattern by -3.0 pA continuous current injection (left), and to the regular firing pattern by +1.0 pA continuous current injection (CV = 0.55, right).

Fig. 1-3. NVT Kiss1 neurons in the non-breeding condition show lower firing activities than those in the breeding condition. A-B. Fluorescence of GFP-labeled NVT neurons in the

breeding condition (A) and in the non-breeding condition (B). The Both fluorescence photomicrographs were taken at the same exposure time (1.1 s), gain, intensity, and were processed at the same level of brightness and contrast adjustment using Adobe Photoshop CS6**.** Scale bars: 50μm (A-B). C. Proportions of four different firing patterns in the four experimental conditions. In both sexes, the proportions under the breeding condition were significantly different from those under the non-breeding conditions (male: $P < 0.05$ breeding vs non-breeding; female: $P < 0.01$ breeding vs non-breeding; χ^2 test). D. The mean instantaneous spike frequency of Kiss1 neurons in the non-breeding condition was significantly lower than that in the breeding condition (breeding: 0.8 ± 0.1Hz, non-breeding: 0.1 ± 0.1 Hz; ***: $P < 0.001$ breeding vs. non-breeding; Mann-Whitney *U* test). E. The average RMP of NVT Kiss1 neurons in the breeding condition was -58.2 ± 1.2 mV (n = 58), and was -61.3 ± 1.3 mV (n = 27) in the non-breeding condition (*: $P < 0.05$ breeding vs. non-breeding; Mann-Whitney *U* test).

Fig. 1-4. NVT Kiss1 neurons change their neuronal activities in accordance with estrogen levels. A. Proportions of different firing patterns among the groups of sham operation (Sham), OVX, and OVX + estrogen-treated (OVX+E) female fish. B. Mean instantaneous frequency of Kiss1 neurons from OVX fish is marginally significantly lower than that from OVX+E group, and is rather lower than that from Sham group (Sham: 1.0 ± 0.5Hz, OVX: 0.1 ± 0.1Hz, OVX+E: 0.5 ± 0.1Hz, #: $P < 0.1$ OVX vs. OVX+E; Steel-Dwass test). C. The average RMP of NVT Kiss1 neurons from OVX fish tends to be hyperpolarized compared to that from the OVX+E fish (Sham: -68.0 ± 3.5mV, OVX: -69.1 ± 2.7mV, OVX+E: -59.7 ± 3.3mV; #: $P < 0.1$ OVX vs. OVX+E; Steel-Dwass test).

Table 1-1. The Firing Patterns of NVT Kiss1 Neurons

Supplementary information

On-cell loose-patch clamp recordings

I performed on-cell loose-cell patch-clamp recordings from genetically EGFP-labeled Kiss1 neurons in the daytime (11:00~20:00) by using the *kiss1*: EGFP transgenic medaka. The whole brain *in vitro* preparation of adult male and female transgenic medaka were performed as previously described. The tip resistance of patch pipettes in the ACSF was approximately 10–25 MΩ. Targeted on-cell loose-patch clamp recordings were performed with the Axopatch 200B patch-clamp amplifier, and the action currents, the membrane currents associated with action potential firing, were digitized (10 kHz) and stored on a computer using Digidata 1322A and pCLAMP 9.2 software. ACSF was used for the internal solution, and the seal resistances in the on-cell recordings were about 30 to 80 MΩ. For analyses of loose-patch clamp recordings, I used the recording between 5 and 10 min after the start of stable recordings. I classified the firing activity of Kiss1 neurons into four different firing patterns as follows. Burst firing; three consecutive action currents at intervals less than 500 msec are showed. Regular firing: the CV of the inter-spike interval was less than 0.65. Silent: 1-min bin showed one action potential or less. Irregular firing: recordings that did not match any of the three definitions above. Statistical analyses were performed with Kyplot5 (Kyence, Tokyo, Japan).

Supplemental Fig.1-1. Firing activity of medaka NVT Kiss1:GFP neurons under breeding conditions using loose-cell current-clamp recordings. A. Representative traces of spontaneous firing from NVT Kiss1 neurons. NVT Kiss1 neurons showed bursting, regular, irregular, and silent firing patterns. B. Proportions of different firing patterns in on-cell loose-patch recordings and whole-cell recordings from male and female. Under on-cell loose-patch recordings, NVT Kiss1 neurons from male and female medaka also showed various firing patterns: bursting, 15%; regular, 8%; irregular, 46%; and silent, 31% (n = 13), which was similar to whole-cell recordings: bursting, 13%; regular, 21%; irregular, 45%; and silent, 21% (n =58). There were no significant

differences in the relative proportion of firing patterns between loose-cell current-clamp recordings and whole-cell recordings (P=0.69; χ^2 test).

Fig. 1-1

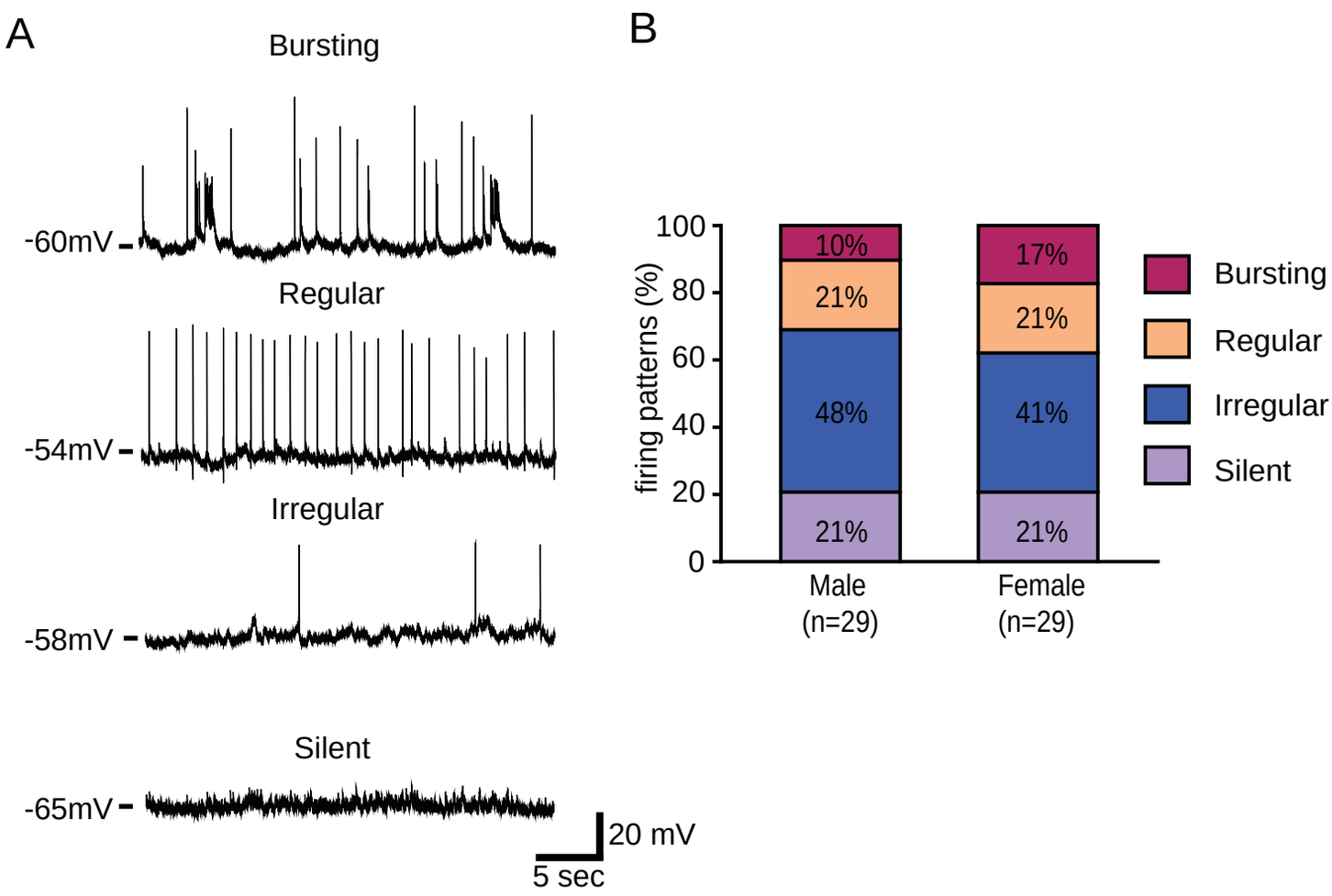

A
Bursting
-60mV
Regular
-54mV
Irregular
-58mV
Silent
-65mV
20 mV
5 sec
B
firing patterns (%)
100
80
60
40
20
0
10%
21%
48%
21%
17%
21%
41%
21%
Male
(n=29)
Female
(n=29)
Bursting
Regular
Irregular
Silent

Fig. 1-2

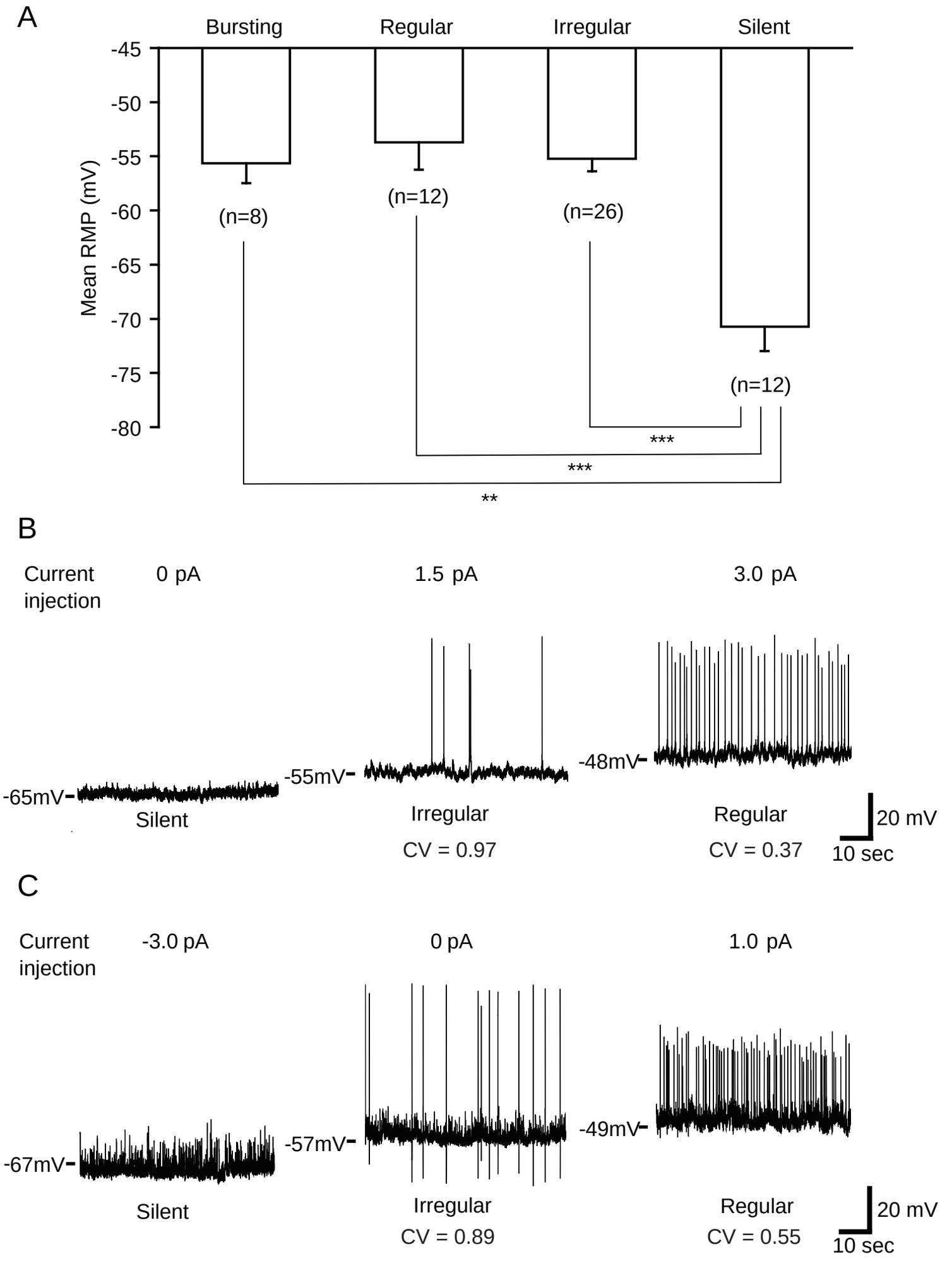

A
Bursting
Regular
Irregular
Silent
Mean RMP (mV)
-45
-50
-55
-60
-65
-70
-75
-80
(n=8)
(n=12)
(n=26)
(n=12)
***
***
**
B
Current injection
0 pA
1.5 pA
3.0 pA
-65mV
-55mV
-48mV
Silent
Irregular
CV = 0.97
Regular
CV = 0.37
20 mV
10 sec
C
Current injection
-3.0 pA
0 pA
1.0 pA
-67mV
-57mV
-49mV
Silent
Irregular
CV = 0.89
Regular
CV = 0.55
20 mV
10 sec

Fig. 1-3

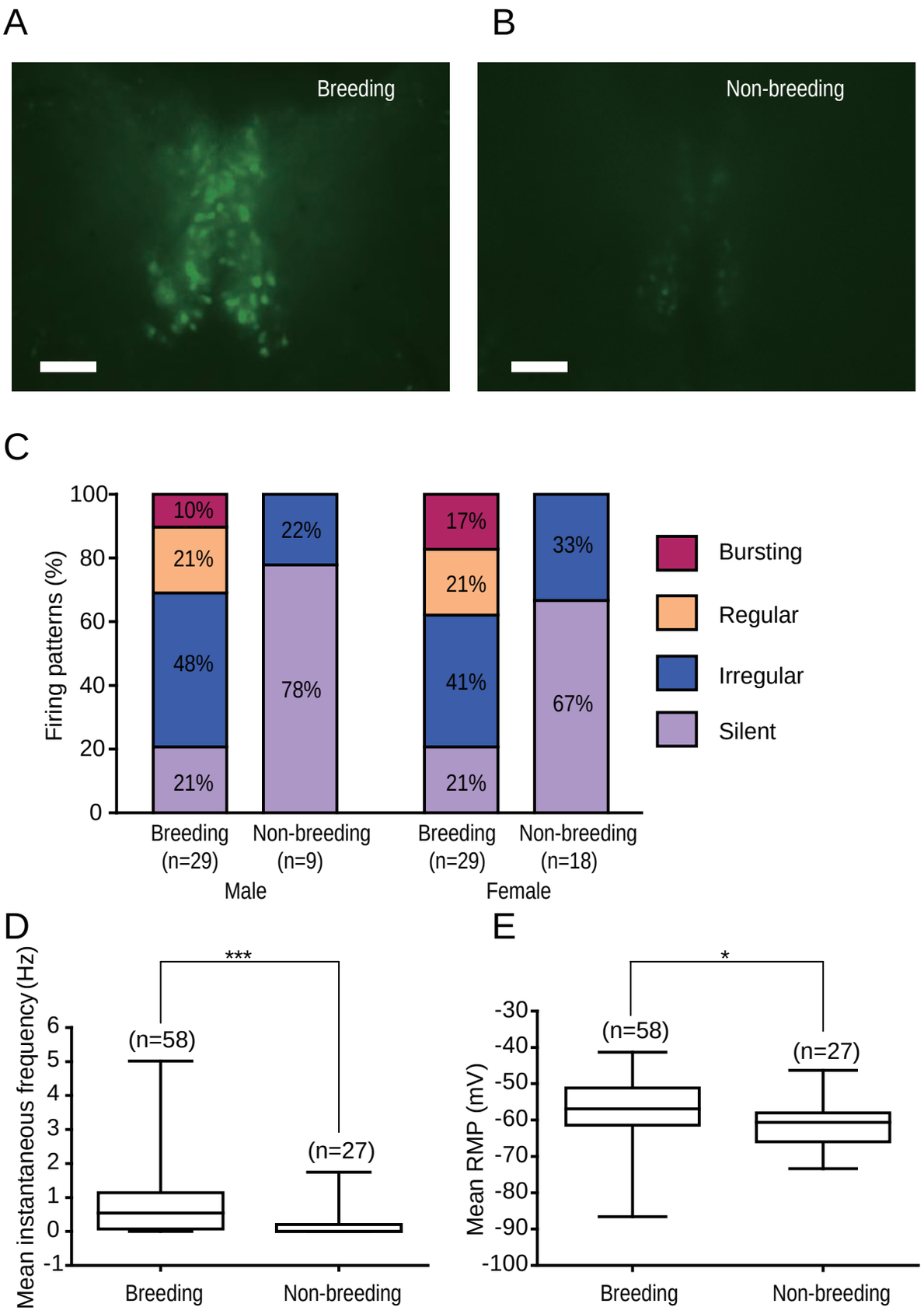

A
B
Breeding
Non-breeding
C
Firing patterns (%)
100
80
60
40
20
0
10%
21%
48%
21%
22%
78%
17%
21%
41%
21%
33%
67%
Bursting
Regular
Irregular
Silent
Breeding (n=29)
Non-breeding (n=9)
Breeding (n=29)
Non-breeding (n=18)
Male
Female
D
Mean instantaneous frequency (Hz)
***
(n=58)
(n=27)
Breeding
Non-breeding
E
Mean RMP (mV)
*
(n=58)
(n=27)
Breeding
Non-breeding

Fig. 1-4

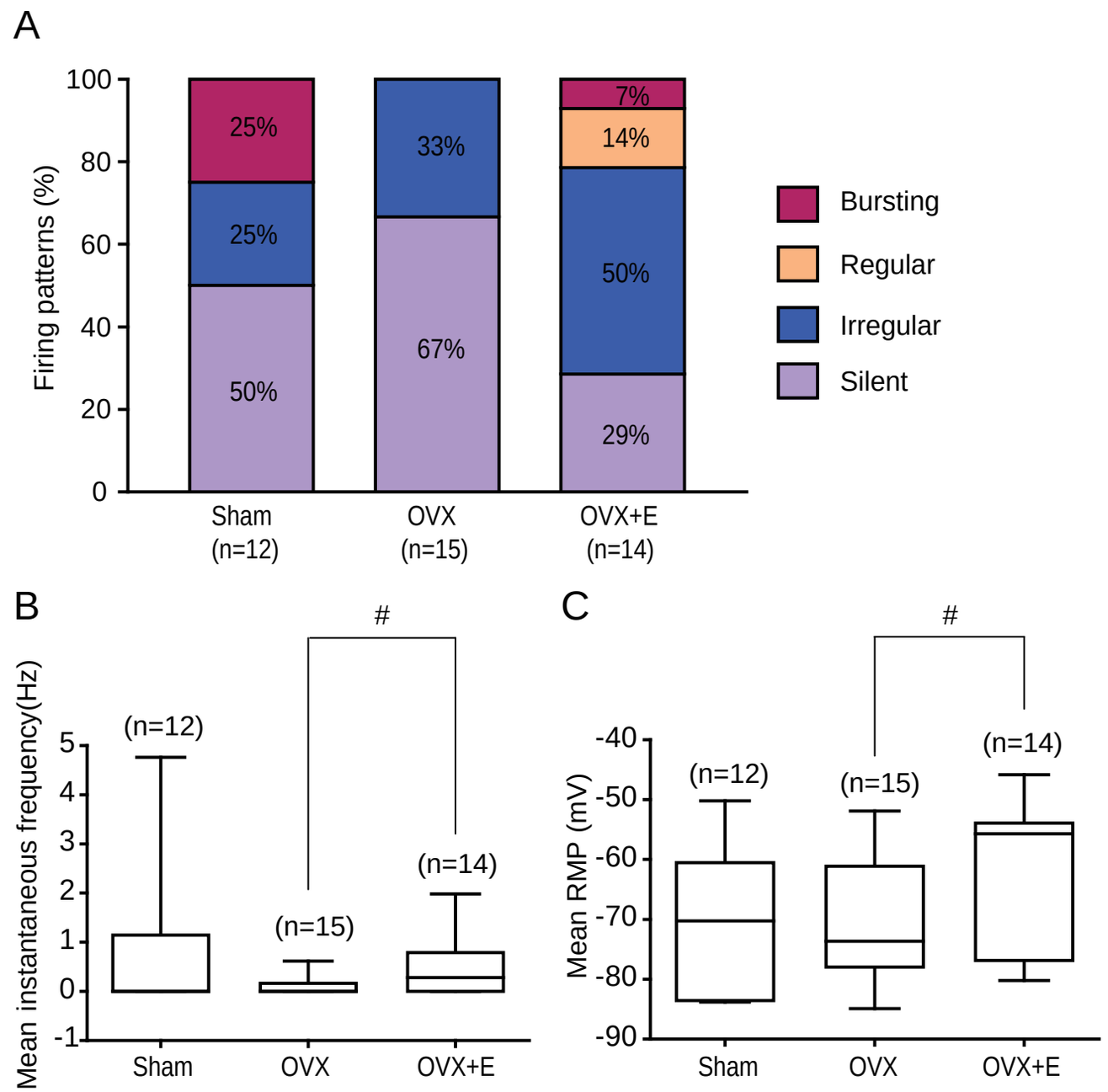
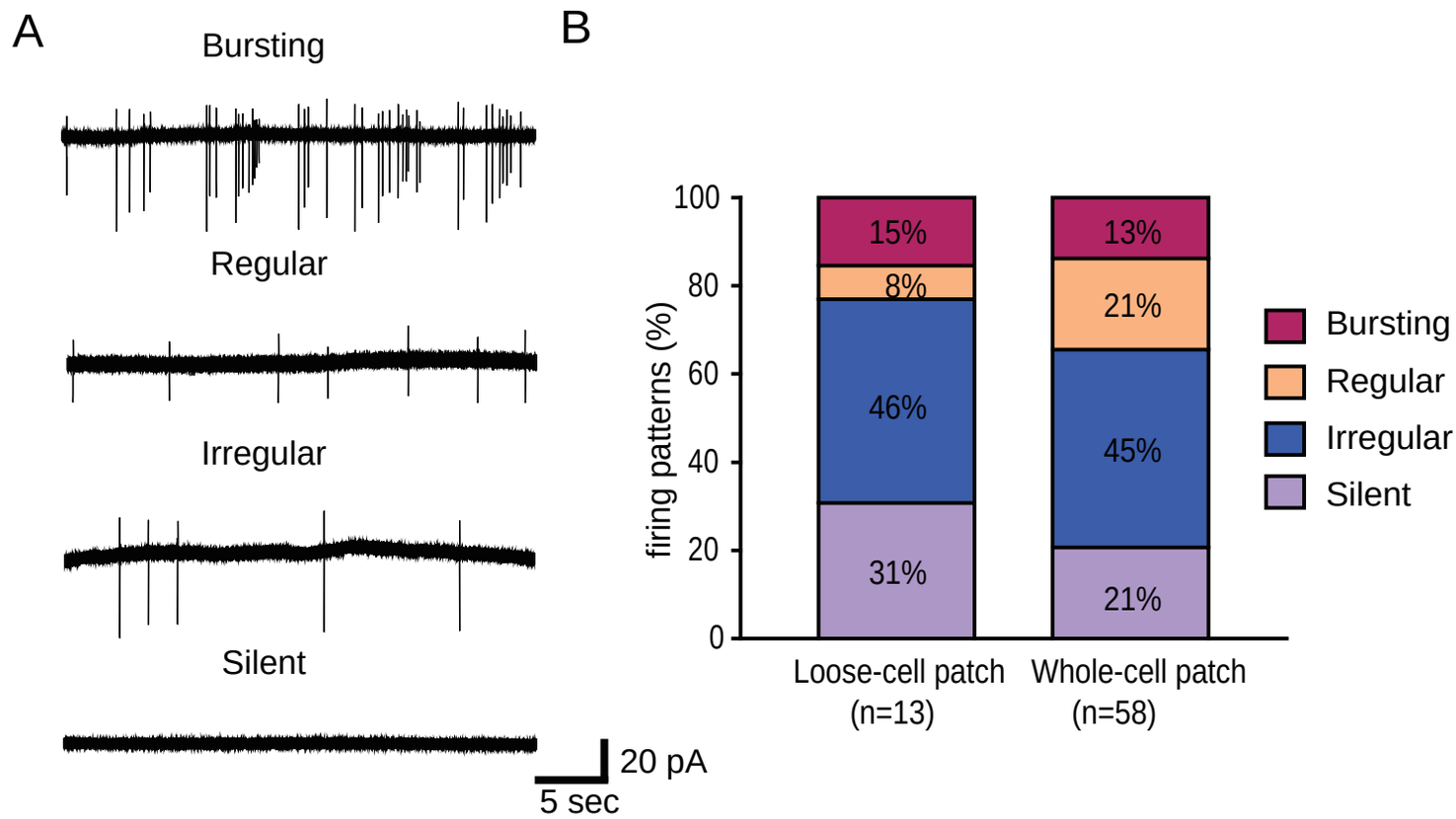

A
Firing patterns (%)
100
80
60
40
20
0
25%
25%
50%
33%
67%
7%
14%
50%
29%
Sham
(n=12)
OVX
(n=15)
OVX+E
(n=14)
Bursting
Regular
Irregular
Silent
B
Mean instantaneous frequency(Hz)
5
4
3
2
1
0
-1
(n=12)
(n=15)
(n=14)
#
Sham
OVX
OVX+E
C
Mean RMP (mV)
-40
-50
-60
-70
-80
-90
(n=12)
(n=15)
(n=14)
#
Sham
OVX
OVX+E

Table 1-1

	Bursting	Regular	Irregular	Silent	Total
Male					
Breeding	3 (10%)	6 (21%)	14 (48%)	6 (21%)	29
Nonbreeding	0 (0%)	0 (0%)	2 (22%)	7 (78%)	9
Female					
Breeding	5 (17%)	6 (21%)	12 (41%)	6 (21%)	29
Nonbreeding	0 (0%)	0 (0%)	6 (33%)	12(67%)	18
Sham	3 (25%)	0 (0%)	3 (25%)	6 (50%)	12
OVX	0 (0%)	0 (0%)	5 (33%)	10 (67%)	15
OVX+E	1 (7%)	2 (14%)	7 (50%)	4 (29%)	14

Supplemental Fig. 1-1.



Chapter 2

Female-specific glucose sensitivity of GnRH1 neurons leads to sexually dimorphic inhibition of reproduction by fasting in medaka

Abstract

Close interaction exists between energy-consuming reproduction and nutritional state. However, there are differences in costs and priority for reproduction among species and even between sexes, which leads to diversification of interactions between reproduction and nutritional state. Despite such diversified interactions among species and sexes, most of the analysis of the nutritional state-dependent regulation of reproduction has been limited to an endothermic vertebrate, mammalian species of either sex. Therefore, the mechanism underlying the diversified interactions remain elusive. In this chapter, I demonstrated the effects of malnutritional state on reproduction at both organismal and cellular levels in an ectothermic vertebrate, a teleost medaka of both sexes. First, I analyzed the effects of malnutrition by fasting on gonadosomatic index, number of spawned/fertilized eggs, and courtship behavior. Fasting strongly suppressed reproduction in females, but surprisingly, not in males. Next, I analyzed the effects of fasting on spontaneous firing activity of hypothalamic gonadotropin-releasing hormone (GnRH1) neurons, which form the final common pathway for the control of reproduction. Electrophysiological analysis showed that low glucose, which is induced by fasting, directly suppresses the spontaneous firing activity of GnRH1 neurons specifically in females through intracellular K_{ATP} channels and AMPK pathways. Based on the fact that such suppressions occurred only in females, I conclude that nutritional state-dependent glucose-sensing in GnRH1 neurons may contribute to the most fitted reproductive regulation for each sex.

Introduction

There is no doubt that reproduction, which is energy consuming, is strongly related to nutritional state. However, strategy for the fittest energy consumption of reproduction is dependent on ecology and physiology of species, and is thus diverse. Interestingly, in various species, there are marked differences even between male and female in the priority for reproductive functions/behaviors, which may be related to the sexual differences in the cost for

reproduction (67,68). In spite of being a widely known phenomenon, the physiological mechanism of the nutritional state-dependent regulation of reproduction for each species and sex are largely unknown.

Gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus and preoptic area (POA) are supposed to form the final common pathway for the control of reproduction in vertebrates by stimulating gonadotropin secretion from the pituitary (69-71). As the key factor in the nutritional state-dependent regulation of reproduction, I focused on the hypothalamic GnRH neurons.

Most of the previous studies that analyzed the relationships between reproduction and energy balance used mammals, such as rodents. However, as being endothermic animals, mammals invest most of the ingested energy in maintenance of body temperature and activity (72). Furthermore, virtually all experimental mammals are viviparous, which is not common throughout vertebrates. Thus, there is a large difference between mammals and other vertebrates in the energy demand/consumption for reproduction. Moreover, there has been virtually no study that focused on the sexual difference. Therefore, to understand the regulatory mechanism of reproduction dependent on nutritional state, the analysis using both sexes of non-mammalian models are indispensable.

In the present study, I chose a non-mammalian model, medaka (*Oryzias latipes*) for this analysis. Because sexually mature medaka spawn every day under favorable breeding conditions, the effects of nutritional state on reproduction can be analyzed easily. Moreover, the brain of medaka is small and transparent, which enables the electrophysiological analysis of neuronal activity using a whole-brain *in vitro* preparation immediately after sacrifice (7,47). By using this fresh whole-brain *in vitro* preparation, we can analyze POA GnRH1 neuronal activities in a condition close to *in vivo*, keeping major synaptic connections intact. Furthermore, morphological/physiological studies in medaka have revealed the projections of POA GnRH1 neurons and the physiological effects of GnRH1 peptide on pituitary gonadotrophs (73,74). Here,

by using this advantageous model, I analyzed the effects of fasting on reproduction and the firing activity of the POA GnRH1 neurons in both sexes.

Material and Methods

Animals

All animals were maintained and used in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) and the protocol approved by the Animal Care and Use Committee of the University of Tokyo (permission number: 15–3). Male and female d-rR strain medaka (*Oryzias latipes*, teleost fish), *gnrh1:* EGFP transgenic medaka (7,74) were maintained in pairs under a 14 h light/10 h dark photoperiod (light on at 08:00 and light off at 22:00) at a water temperature of 27 °C. I used the pairs of sexually mature female and male medaka, that were over 3 months of age and continuously spawned.

Analysis of sexual behaviors and reproductive states

For the analysis of the sexual behaviors, I used sexually mature male and female d-rR strain medaka. I prepared six male and female pairs for each group: normally fed, fasted female (normally fed male × fasted female), and fasted male (fasted male × normally fed female) groups. Each pair was kept in a plastic tank (9 cm x 16 cm x 10.5 cm). During three days before the start of fasting, I fed all pairs with live brine shrimp two or three times per day, and confirmed that spawned eggs and courtship behaviors were observed in all pairs [Fig. 2-1, Times (day): -2 to 0]. Then, the males and females were fasted for 14 days in male- and female-fasted groups, respectively. It should be noted that all medaka survived and swam normally after two-week-fasting. The body weight of two-week-fasted medaka was restored by re-feeding (data not shown). Additionally, the pairs of two-week-fasted male and female medaka resumed spawning by re-feeding for 10 days or more (data not shown). These results suggest that two-week-fasting did not have a serious effect on the physiological conditions and behaviors of medaka. The pairs in the normally-fed group, males in

the female-fasting group, and females in the male-fasting group were continuously fed with live brine shrimp two or three times daily. Males in the female-fasting group, and females in the male-fasting group were fed individually in isolated tanks to avoid feeding fasted female or male. By using a transparent plastic cup with small holes for water exchange, I separated male and female at night (18:00-20:00). In the next morning (10:00~11:00), I removed the transparent cup to make a breeding pair. I counted the spawned/fertilized eggs in all pairs one hour after removal of the transparent cup. For the analysis of fasting-effect on the courtship behaviors, I counted the frequency of "quick-circle", which is known as a courtship behavior of male medaka (75). I recorded and counted the courtship behaviors for 10 min after the removal of the transparent cup using a digital video recorder (HDR-SR7 or HDR-CX420, Sony, Tokyo, Japan). After the 14-day fasting experiment, I sacrificed the female by deep anesthesia (0.02 % tricaine methanesulfonate, MS-222; Sigma-Aldrich, St. Louis, MO), and measured the body-weight and the gonad-weight, to calculate the gonad somatic index (GSI): (gonad-weight / body-weight) x 100. For the analysis of the body weight, I prepared six pairs of mature male and female d-rR strain medaka for normally fed and fasted groups. I measured their body weight before the start of fasting period (before) and after the 2-week-fasting period (after), and calculated the ratio of body weight (after/before).

Electrophysiology

For the electrophysiological analysis of GnRH1 neuronal activities, I used the *gnrh1:* EGFP transgenic line, which specifically label GnRH1 neurons by EGFP (7,74). I recorded the firing activity of GnRH1 neurons using whole brain *in vitro* preparations based on a previous study (7). Briefly, medaka were anesthetized by immersion in 0.02% MS-222 and decapitated. The whole brain was rapidly placed in a hand-made chamber filled with artificial cerebrospinal fluid (ACSF). The ACSF contained 134 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES. Additionally, based on results of blood glucose measurement in normally-fed and 2-week-fasted medaka, I added 4.5 mM glucose (standard glucose condition) or 0.5 mM glucose (fasting-like

condition) to ACSF (finally adjusted to pH 7.4 with NaOH, and osmolality was adjusted to approximately 295–300 mOsm/liter with sucrose). Then, the optic nerve and meningeal membrane was peeled off. GnRH1: EGFP neurons were easily identified by their fluorescence under an upright fluorescent microscope with infrared-differential interference contrast optics (Eclipse E-600FN; Nikon, Tokyo, Japan). The patch pipettes were made from borosilicate glass capillaries of 1.5 mm outer diameter (GD-1.5; Narishige, Tokyo, Japan) by using a Flaming-Brown micropipette puller (P-97; Sutter Instruments, Novato, CA). Targeted on-cell loose-patch and whole-cell patch recordings were performed with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale CA). All recordings were digitized (10 kHz) and stored on a computer using Digidata 1322A and pCLAMP 9.2 software (Molecular Devices). The tip resistance of patch pipettes in an ACSF was approximately 10–15 MΩ for on-cell loose-patch recordings, and 15-30 MΩ for whole-cell patch recordings. I used ACSF as the pipette solution for loose-patch recordings, and a loose seal (approximately 40 to 100 MΩ seal resistance) was formed to record spontaneous action currents of GnRH1 neurons in the voltage clamp mode. For whole-cell patch clamp recordings, I used a pipette solution containing 112.5 mM K^+-gluconate, 4.0 mM NaCl, 17.5 mM KCl, 1.0 mM $MgCl_2$, 1 mM EGTA, 10 mM sucrose, 10 mM HEPES, and 0.5 mM $CaCl_2$ (pH 7.2, adjusted with KOH). The membrane potential including the spontaneous firing of GnRH1 neurons was recorded in the current clamp mode. Access resistance was less than 300 MΩ in whole-cell configuration. For all analyses, the liquid junction potential was corrected off-line.

Experimental design of electrophysiology

I analyzed the electrophysiological data by using clampfit10.2 (Molecular Devices). Because the previous study suggested that spontaneous firing activity of the POA GnRH1 neurons of female medaka shows time-of-day-dependent changes (7), I recorded the spontaneous firing activity in the evening period (16:00~20:00), when GnRH1 neurons show highest activity in a day. I calculated the instantaneous frequency and the number of firings from the recording data between 5 and 10

mins after the start of recordings, as described in the previous report (7).

Most of the recordings were performed in the 4.5 mM glucose ACSF (normal ACSF), unless otherwise noted. To analyze the effect of 0.5 mM glucose ACSF (Fig. 2-3) or the activators of K_{ATP} channels and AMPK in whole-cell patch recordings, ACSF containing 0.5 mM glucose or the activators were perfused for 10 min after 10 min-recordings in the normal ACSF. After the perfusion of these solutions for 10 min, I restored the perfusing solution to 4.5 mM glucose ACSF. To examine the effects of blockers of intracellular signaling on 0.5 mM ACSF-induced inhibition of firing activity in female GnRH neurons, 0.5 mM glucose ACSF was applied for 10 min in the presence of the blockers. The mean frequency or the number of firings in five minutes were calculated between 5 and 10 min after the start of perfusion of the different solution (during), and during 5-min periods before the start of the perfusion (before). The relative frequency and the relative number of firings were defined as [mean frequency or number of firings (during) / mean frequency or number of firings (before)].

Drugs

Diazoxide, and tolbutamide were purchased from Sigma (St. Louis, MO). Dorsomorphin (Compound C), and 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) were obtained from Wako (Osaka, Japan). A stock solution of diazoxide (50 mM) was prepared in 0.1N NaOH. Stock solutions of tolbutamide (400 mM) and dorsomorphin (10 mM) were prepared in dimethyl sulfoxide. A stock solution of AICAR (20 mM) was prepared in ACSF.

Analysis of blood glucose level

I used sexually mature male and female d-rR strain medaka for the analysis of blood glucose level. I prepared normally-fed and two-week-fasted medaka. After anesthesia by immersion in 0.02% MS-222, the medaka's heart was exposed by dissection, and blood was collected from the conus anteriosus by a borosilicate glass pipette made from capillaries of 1.0 mm outer diameter

(GD-1.0; Narishige, Tokyo, Japan), which contained heparin sodium salt (Wako, Osaka, Japan). The collected blood samples were centrifuged, and serum was collected. I applied 5μL Somogyi's copper reagent (Sigma-Aldrich, St. Louis, MO) to 1μL plasma sample, and boiled it at 100 °C for 10 min. Then, the solutions were cooled on an ice bath for several minutes. Next, 5μL Nelson color reagent (Sigma-Aldrich, St. Louis, MO) was added, and the solutions were vortexed. After centrifugation of the solutions, I collected the supernatant and measured the absorbance at 650 nm by NanoDrop ND-1000 (MIT BioMicroCenter, Cambridge, MA). The glucose level of each sample was calculated by the standard curve method.

Statistics

Statistical analyses were performed with Kyplot5 software (Kyence, Tokyo, Japan). In the data for the statistical analysis, I confirmed the normal distribution by using the Shapiro-Wilk test. Additionally, the homoscedasticity was also confirmed by F test in the comparisons between the two groups. For the normally distributed and homoscedastic data, I used the parametric tests, as described in Results (e.g., Fig. 2-1B, unpaired two-tailed *t*-test, Fig. 2-4E, Tukey test for multiple comparison). For the data with non-normal distribution, I performed the non-parametric tests (e.g., Fig. 2-1A, Mann-Whitney *U* test, Fig. 2-4B, Steel-Dwass test for multiple comparison). All values are shown as mean ± SEM. For all statistics, significance levels were set as $P < 0.05$. No statistical method was used to predetermine sample sizes, but the sample sizes are similar to previous reports (7,76). Pairs of sexually mature male and female medaka were assigned to each experimental group without randomization or blinding.

Results

Fasting strongly suppresses reproduction in female, but not in male

I analyzed the effect of fasting on the reproduction of mature female and male d-rR strain medaka. In females, the number of eggs spawned was significantly reduced by fasting for three days or more

(Fig. 2-1A; $P < 0.01$, Mann-Whitney *U* test). Furthermore, the GSI of 2-week-fasted females was significantly lower than that of normally fed females (Fig. 2-1B; Normally fed: 7.0 ± 0.4, 2-week-fasting: 0.9 ± 0.2, $P < 0.001$, unpaired two-tailed *t*-test).

Next, I analyzed the effect of fasting on the reproductive function and behavior in males. Surprisingly, the number of fertilized eggs in pairs of 2-week-fasted male and normally fed female did not show any decrease (Fig. 2-1C, Mann-Whitney *U* test). Furthermore, there was no significant difference between the normally fed and fasted males in the number of courtships to normally fed females (Fig. 2-1D, Mann-Whitney *U* test). On the other hand, 2-week fasting significantly decreased the body weight in males similar to that in females (Fig. 2-1E; Fed female; 1.05 ± 0.05, Fasted female; 0.81 ± 0.01, Fed male; 1.02 ± 0.02, Fasted male; 0.83 ± 0.02, n=6, $P < 0.001$ Fed male; Fed female vs Fasted male; Fasted female, Tukey-test).

These results suggest that fasting strongly inhibits reproduction of females, but not that of males.

GnRH1 neurons in female, but not in male, show low spontaneous firing activity in the fasting-induced low glucose condition

To examine the effects of fasting on the spontaneous firing activity of GnRH1 neurons, I analyzed firing activity of GnRH1 neurons in the normally fed (Fed) and 2-week-fasted (Fasted) fish using on-cell loose-patch clamp recording.

First, I compared the spontaneous firing activity of GnRH1 neurons between Fed and Fasted females recorded in a standard extracellular solution containing 4.5 mM glucose. Fig. 2-2A shows typical traces of spontaneous firing activity of GnRH1 neurons in the Fed and Fasted females in 4.5 mM glucose. Interestingly, there was no significant difference in the mean instantaneous frequency (Fig. 2-2B; Fed: 2.7 ± 0.7, n = 16, Fasted recorded in 4.5 mM glucose: 2.0 ± 0.7, n =13, Steel-Dwass test) and the number of firings (Fig. 2-2C; Fed: 475.1 ± 127.6, n = 16, Fasted recorded in 4.5 mM glucose: 241.2 ± 50.3, n=13, Steel-Dwass test).

A variety of glucose-sensing neurons, which change their neuronal activity according to the

glucose level, have been reported in the brain (77). Therefore, in addition to chronic effects, I examined acute effects of low glucose by adjusting the glucose concentration of ACSF to physiological level of Fasted medaka. First, I measured blood glucose level of 2-week-fasted medaka and found that it was much lower than that of normally fed medaka (Fed: 3.5 ± 0.9 mM, Fasted: 0.6 ± 0.2 mM, n = 6, $P < 0.01$, Mann–Whitney *U* test). By recording firing activity of GnRH1 neurons in Fasted female in a low glucose (0.5 mM glucose) condition, I demonstrated that GnRH1 neurons of Fasted females in the low glucose condition show significantly lower mean instantaneous frequency as well as fewer number of firings in 5 mins than those of Fed and Fasted females in a standard glucose level (Fig. 2-2B; Fasted in 0.5 mM glucose: 0.8 ± 0.3, n =19, $P < 0.01$ Fed vs Fasted recorded in 0.5 mM glucose, $P < 0.05$ Fasted recorded in 4.5 mM glucose vs Fasted recorded in 0.5 mM glucose, Steel-Dwass test; Fig. 2-2C; Fasted recorded in 0.5 mM glucose: 133.4 ± 65.0, n =19, $P < 0.01$ Fed vs Fasted recorded in 0.5 mM glucose, $P < 0.05$ Fasted recorded in 4.5 mM glucose vs Fasted recorded in 0.5 mM glucose, Steel-Dwass test).

I also analyzed firing activity of GnRH1 neurons in normally-fed (Fed) and 2-week fasted (Fasted) male. In males, GnRH1 neurons of the 2-week-fasted group in the low glucose recording condition showed similar firing activity to that in normally-fed condition (Fig. 2-2D). There was no significant difference in the mean instantaneous frequency (Fig. 2-2E; Fed: 1.7 ± 0.3, n = 15, Fasted recorded in 0.5 mM glucose: 1.8 ± 0.3, n =14, unpaired two-tailed *t* test) and the number of firings (Fig. 2-2F; Fed: 326.5 ± 50.0, n = 15, Fasted recorded in 4.5 mM glucose: 277.4 ± 37.4, n=14, unpaired two-tailed *t* test).

These results suggest that spontaneous firing activity of GnRH1 neurons in females, but not in males, is low in the fasting condition through acute effects of low glucose in the cerebrospinal fluid.

Low glucose directly inhibits the firing activity of GnRH1 neurons in female, but not in male

As shown in Fig. 2-2A, in 2-week fasted females, spontaneous firing activity of GnRH1 neurons in a low glucose (0.5 mM) ACSF was low, whereas that in a standard glucose (4.5 mM) ACSF was

similar to that in the normally-fed group. Therefore, I next analyzed the effect of an acute change of glucose level on neuronal activity of GnRH1 neurons, using whole cell patch clamp recording.

Firing activity of GnRH1 neurons in females was acutely suppressed by changing the glucose level of ACSF from 4.5 mM to 0.5 mM (Fig. 2-3A). To examine whether GnRH1 neurons themselves are the glucose-sensing neurons, I also analyzed the acute effect of glucose level on neuronal activity of GnRH1 neurons using patch pipette solution containing 3 mM glucose, in which intracellular glucose concentration of the recorded GnRH1 neuron was maintained, while the other neurons are affected by low glucose ACSF. In the 4.5mM extracellular glucose condition before change to 0.5mM glucose, the spontaneous firing activity of GnRH1 neurons with 3mM intracellular glucose is similar to that without intracellular glucose. A representative recording with 3 mM glucose-containing pipette solution did not show changes in firing activity of GnRH1 neurons by low glucose ACSF (Fig. 2-3B). Fig. 2-3C shows relative frequencies of GnRH1 firing among three groups (4.5 mM extracellular glucose, 0.5 mM extracellular glucose, and 0.5mM extracellular glucose with 3 mM intracellular glucose). The relative frequency in 0.5 mM extracellular glucose was significantly lower than that in 4.5 mM extracellular glucose , while that in 4.5 mM extracellular glucose was not significantly different from that in 0.5 mM extracellular glucose with 3 mM intracellular glucose (4.5 mM extracellular glucose ; 1.4 ± 0.4, 0.5 mM extracellular glucose ; 0.4 ± 0.1, 0.5 mM extracellular glucose with 3 mM intracellular glucose ; 0.8 ± 0.1, n =7, 4.5 mM extracellular glucose vs 0.5 mM extracellular glucose , $P < 0.05$, Steel-Dwass test). The relative number of firings in 0.5 mM extracellular glucose, but not in 0.5 mM extracellular glucose with 3 mM intracellular glucose, was also significantly lower than that in 4.5 mM extracellular glucose (Fig. 2-3D; 4.5 mM extracellular glucose; 1.2 ± 0.4, 0.5 mM extracellular glucose; 0.3 ± 0.1, 0.5 mM extracellular glucose with 3 mM intracellular glucose; 0.7 ± 0.1, n =7, $P < 0.05$, 4.5 mM extracellular glucose vs 0.5 mM extracellular glucose, Steel-Dwass test).

In males, however, the change of glucose level did not affect neuronal activity of GnRH1 neurons (Fig. 2-3E). There was no significant difference between the 4.5 mM and 0.5 mM extracellular

glucose in the relative frequency (Fig. 2-3F; 4.5 mM extracellular glucose ; 0.9 ± 0.3, n =8, 0.5 mM extracellular glucose ; 1.1 ± 0.2, n =10, Mann–Whitney *U* test) and the relative number of firings (Fig. 2-3G; 4.5 mM extracellular glucose; 0.8 ± 0.4, n =8, 0.5 mM extracellular glucose; 1.0 ± 0.2, n =10, Mann–Whitney *U* test).

These results suggest that the low glucose level directly inhibits the neuronal activity of GnRH1 neurons in females, but not in males.

Activators of K_{ATP} channels and AMPK inhibit GnRH1 firing activity in females, but not in males

Previous studies reported that ATP-sensitive potassium (K_{ATP}) channels play essential roles in modulation of various cellular activities in the brain and peripheral tissues related to the glucose level (78-80) . I examined whether the K_{ATP} channels modulate GnRH1 neuronal activity by using a K_{ATP} channels activator, diazoxide (500 μM). Fig. 2-4A shows that the bath application of diazoxide inhibited the spontaneous firing activity of GnRH1 neurons in females. The perfusion of diazoxide decreased the instantaneous frequency (Fig. 2-4B, n=6, $P < 0.1$, Before vs Diazoxide, Steel-Dwass test) and the number of firings (Fig. 2-4C, n=6, $P < 0.05$, Before vs Diazoxide, Steel-Dwass test). I also analyzed the relationship between GnRH1 neuronal activity and AMP-activated protein kinase (AMPK). AMPK has been reported to be activated by a shift of intracellular AMP/ATP ratio related to the change of glucose level and modulates the neuronal activities (76,81,82). To examine whether this AMPK is involved in the modulation of the GnRH1 firing activity, I analyzed the effect of an AMPK activator, AICAR on the GnRH1 firing activity. The perfusion of 2 mM AICAR suppressed the firing activity of GnRH1 neurons in female (Fig. 2-4D). Although the effect of AICAR did not completely recover after wash-out, the application of AICAR reduced the instantaneous frequency (Fig. 2-4E, n=6, $P < 0.05$, Before vs AICAR; Wash-out, Tukey-test) and the number of firings (Fig. 2-4F, n=6, $P < 0.05$, Before vs AICAR; Wash-out, Tukey-test). On the other hand, in males, neither diazoxide nor AICAR had any significant effect on the GnRH1 firing activity (Fig. 2-4G-I, n=8; Fig. 2-4J-L, n=8, respectively, Steel-Dwass test).

Thus, the activators of K_{ATP} channels and AMPK inhibit GnRH1 firing activity in females, but not in males.

The glucose response of GnRH1 neurons is impaired in the presence of blockers for K_{ATP} channels and AMPK

In the previous section, activators of K_{ATP} channels and AMPK showed the female-specific inhibitory effect on GnRH1 firing activity similar to the low glucose. I therefore hypothesized that the female-specific glucose response in GnRH1 neurons is mediated by K_{ATP} channels and AMPK. To verify this hypothesis, I examined the low-glucose induced suppression of the GnRH1 firing activity in the presence of a K_{ATP} channel blocker, tolbutamide, and/or an AMPK blocker, dorsomorphin. Neither tolbutamide (200μM) nor dorsomorphin (20μM) blocked the low-glucose induced suppression of GnRH1 firing activity (Fig. 2-5A, B). On the other hand, in the presence of both tolbutamide and dorsomorphin, the low-glucose induced inhibition of GnRH1 firing activity was abolished (Fig. 2-5C). In the 4.5 mM glucose ACSF, the blockers did not show significant effect on the relative frequency (Fig. 2-5D; ACSF only; 1.7 ± 0.5, n=9, tolbutamide; 1.3 ± 0.2, n=10, dorsomorphin; 2.7 ± 1.6, n =6, tolbutamide + dorsomorphin; 0.7 ± 0.1, n=8) or the relative number of firings (Fig. 2-5E; ACSF only; 0.8 ± 0.1, n=9, tolbutamide; 1.2 ± 0.3, n=10, dorsomorphin; 1.6 ± 0.9, n =6, tolbutamide + dorsomorphin; 0.8 ± 0.1, n=8). In the presence of tolbutamide or dorsomorphin, the relative frequency for the 0.5 mM glucose ACSF was low, whereas, in the presence of both, that for 0.5 mM glucose ACSF was high (Fig. 2-5D; ACSF only; 0.4 ± 0.1, n=9, tolbutamide; 0.5 ± 0.1, n=10, dorsomorphin; 0.8 ± 0.4, n =6, tolbutamide + dorsomorphin; 1.1 ± 0.2, n=8). Similar results were obtained for the relative number of firings (Fig. 2-5E; ACSF only; 0.4 ± 0.1, n=9, tolbutamide; 0.5 ± 0.1, n=10, dorsomorphin; 0.6 ± 0.3, n =6, tolbutamide + dorsomorphin; 1.0 ± 0.2, n=8).

These results suggest that both K_{ATP} channels and AMPK are involved in the acute inhibition of GnRH firing activity induced by low glucose in female medaka.

Discussion

In this chapter, I analyzed the effects of fasting-induced malnutrition on reproduction in both sexes of medaka to clarify the relationship between reproduction and nutritional state. Female medaka stopped spawning in malnutritional state by fasting, while long-term fasting had no significant effect on reproductive functions/behaviors in the male medaka. I also found the female-specific glucose-sensitivity of the spontaneous firing activity in POA GnRH1 neurons, which is mediated by K_{ATP} channels and AMPK pathways. These results at organismal and cellular levels suggest that the glucose-sensing in GnRH1 neurons may cause the sexual differences in the regulation of reproduction under malnutritional state.

Firing activity of POA GnRH1 is inhibited by low glucose ACSF, which may contribute to the female-specific fasting-induced suppression of reproduction

The present study showed the suppression of spawning in fasted female (Fig. 2-1A). On the other hand, 2-week fasting had no significant effect on fertility in male (Fig. 2-1C). The histological analysis of testis also demonstrated that spermatogenesis is normal in 2-week fasted male similar to normally fed male (Supplemental Fig.2-1). These results indicate that fasting suppresses reproduction only in females. Previous studies have shown the occurrence of sexual difference in the energy cost for reproduction in various species (67,68), which may be correlated to the sexual differences in the central regulation of reproduction under low energy conditions. Despite such well-known sexual differences in the relationship between reproduction and nutritional state, the underlying mechanisms are poorly understood.

In mammals, the hypothalamic kisspeptin neurons are known to be essential for the reproductive regulation through direct activation of the hypothalamic GnRH neurons (23,69). Recently, the kisspeptin neurons have been demonstrated to receive information about the nutritional state and suggested to be a strong candidate for the link between reproduction and energy balance in

mammals (83,84). However, although the kisspeptin genes are conserved throughout vertebrates including non-mammals (44), recent studies in teleosts suggest that kisspeptin receptors are not expressed in GnRH1 neurons (25), and kisspeptin signaling is dispensable for reproduction (26). On the other hand, the recent study revealed that *gnrh1* knockout medaka are infertile (85), and hypothalamic GnRH neurons are therefore strongly suggested to form the final common pathway for the central regulation of reproduction throughout vertebrates. Therefore, I hypothesized that the energy state directly signals to the hypothalamic GnRH neurons, which contributes to the nutritional state-dependent modulation of reproduction. I then analyzed the effect of fasting on the firing activity of POA GnRH1 neurons in both sexes. The electrophysiological analysis demonstrated that low glucose ACSF, which mimics fasting condition, acutely reduces firing activity of the POA GnRH1 neurons in females, but not in males (Fig. 2-3). It may contribute to the female-specific suppression of reproduction in fasting conditions. In females, the number of spawned eggs significantly decreased starting from day 3 of fasting (Fig. 2-1A). The blood glucose level of 3-day-fasted female was also significantly lower than that of normally fed female (Normally fed female: 4.9 ± 0.9 mM, 3-day-fasted female: 1.4 ± 0.3 mM, n = 4, $P < 0.05$, unpaired two tailed *t*-test). These results support the hypothesis that low glucose-induced suppression of firing activity of GnRH1 neurons leads to inhibition of reproduction in females.

Hypothalamic neurons in mammals, such as orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons in the arcuate nucleus (ARC), integrate various energy-signals and control food intake and energy-metabolism (86,87). Previous studies in rodents reported that the orexigenic and anorexigenic neuropeptides have a variety of effects on GnRH neuronal activities (88-91). In the present study, by using whole-brain in *vitro* preparation, I could analyze the GnRH1 firing activity without disrupting major synaptic inputs from other neurons to GnRH1 neurons. Surprisingly, there was no significant difference between normally fed and 2-week-fasted female in the GnRH1 firing activity under a standard glucose recording condition (Fig. 2-2A-C). These data suggest that fasting does not have

chronic effects on spontaneous GnRH1 firing activity, which is at least much weaker than acute suppression by low glucose ACSF. In addition to these hypothalamic neurons, other peripheral metabolic signals, such as leptin and adiponectin secreted from the adipocyte, have been reported to directly or indirectly act on the hypothalamic GnRH neurons in mammals (92,93). Future analysis of these peripheral hormones/peptides on GnRH1 neuronal activities is important for the better understanding of the nutritional state-dependent modulation of reproduction.

Mechanisms of sexual dimorphism in glucose-sensing of GnRH1 neurons

Hypothalamic GnRH1 neurons of female medaka showed glucose-sensing in the firing activity (Fig. 2-3A-D). In addition to hypothalamic GnRH1 neurons, it has been reported that there are two non-hypothalamic GnRH systems (midbrain GnRH2 and terminal nerve GnRH3) in many vertebrate species (21,70) . Based on physiological, morphological, and molecular biological studies, each GnRH system is suggested to function differently. Because non-hypothalamic GnRH3 neurons do not show such glucose concentration-dependent changes in firing (data not shown), this property is likely to be a characteristic of hypothalamic GnRH neurons. The physiological analysis in the rodent models also showed the glucose-sensing of hypothalamic GnRH neurons (76,94). Based on the results of the present study in a non-mammalian model and these previous reports in mammals, the glucose-sensing of hypothalamic GnRH neurons may be conserved throughout vertebrates. On the other hand, the male medaka did not show glucose-sensing of POA GnRH1 neurons (Fig. 2-3E-G). The activators of K_{ATP} channels and AMPK, which are reported to be involved in glucose-sensing, inhibited GnRH1 firing activity in females, but not in males (Fig. 2-4). Thus, the sexual differences in the activities of K_{ATP} channels and AMPK may contribute to the female-specific glucose-sensitivity of POA GnRH1 neurons. Although the factors that cause the sexually dimorphic properties of GnRH1 neurons are not completely understood, the previous studies in some cells, such as GnRH neurons and pancreatic cells in rodents, showed that sex steroid hormones, 17β-estradiol and dihydrotestosterone, modulate the glucose-sensing or the K_{ATP} channel

activity (76,94-97). Therefore, it is possible that these sex steroid hormones mediate the sexual dimorphism in glucose-sensing of POA GnRH1 neurons. I preliminary examined the chronic effects of gonadal steroids on glucose sensitivity in adults by using females under a non-reproductive condition and found no changes in glucose sensitivity of GnRH1 neurons (Supplemental Fig.2-2). Thus, the sexual difference is likely to be the organizational effects of sex steroid hormones or due to a sexual difference that is independent of gonadal steroids (98,99). The verification of these possibilities may be an interesting future topic to elucidate sexual differences in neurons in general.

Intracellular signaling pathway of glucose-sensing in GnRH1 neurons

The intracellular glucose metabolism linked to K_{ATP} channels and/or AMPK has been reported to mediate glucose-sensing in a variety of neurons (76,78,79,81,82,100,101). On the other hand, a previous study in *Drosophila* reported that a G protein-coupled receptor, bride of sevenless (BOSS) responds to extracellular glucose and regulates intracellular signaling (102). I examined whether the intracellular glucose metabolism is involved in the glucose-sensing of POA GnRH1 neurons by using glucose-containing patch pipette solutions, in which the intracellular glucose level was maintained to 3 mM. Low glucose-induced inhibition was abolished by a 3 mM glucose-containing intracellular solution (Fig. 2-3B), which suggests that the glucose-sensing in POA GnRH1 neurons is mediated by the intracellular glucose metabolism, and not by extracellular sensors or changes in synaptic inputs from interneurons. K_{ATP} channels and AMPK are known to be activated according to the level of intracellular glucose metabolism (AMP/ATP ratio) and modulate the excitability of neurons. Therefore, I finally analyzed the role of K_{ATP} channels and AMPK in the glucose-response of POA GnRH1 neurons. Co-application of the blockers for K_{ATP} channels and AMPK, tolbutamide and dorsomorphin, respectively, suppressed the low glucose-induced inhibition of GnRH1 firing activity, whereas single application of tolbutamide or dorsomorphin did not (Fig. 2-5). This may suggest a complementary relationship between K_{ATP} channels and AMPK pathways. Although the present study did not clarify the target of AMPK-modulation, it has been reported that AMPK

modulates the activities of various ion channels, such as voltage-gated potassium channels (103), large conductance Ca^{2+} activated K^+ channels (104,105), voltage-gated sodium channels (106) in addition to K_{ATP} channels (107,108). Because the glucose response in female GnRH1 neurons could not be blocked by the single application of the blocker for K_{ATP} channels, AMPK may mediate the glucose-sensing through modulation of some ion channel activities besides the K_{ATP} channel-activity. Future voltage-clamp analysis of the interaction between K_{ATP} channels and AMPK pathway in the glucose-sensitivity of GnRH1 neurons may solve this problem.

Conclusions

In summary, I showed in female medaka that the final common pathway for the control of reproduction, POA GnRH1 neurons, shows glucose-sensitivity. Previous studies also showed the glucose-sensitivity of hypothalamic GnRH neurons in mammals (76,94), which suggests that the glucose-sensitivity of the hypothalamic GnRH neurons may be conserved throughout vertebrates.

Surprisingly, the present analysis in both sexes of medaka showed that reproduction in females, but not in males, is strongly suppressed by the malnutritional state through fasting. Furthermore, I found that the fasting-induced low glucose ACSF has female-specific inhibitory effects on POA GnRH1 firing activity. These findings suggest that only females possess the central mechanisms to suppress reproduction in accordance with malnutrition state in medaka. On the other hand, a physiological analysis in rodents has demonstrated that males also have the mechanism for glucose-dependent regulation in GnRH neurons (94). Such differences in glucose sensitivity of hypothalamic GnRH neurons may be related to diversified interactions between reproduction and nutritional state among species/sexes. Future analyses of the diversification of glucose-sensing in hypothalamic GnRH neurons may contribute to the understanding of successful reproductive regulation dependent on energy homeostasis of each species.

Figure legends

Fig. 2-1. Fasting inhibits reproduction of mature female but not male medaka. (A) The mean number of eggs spawned for 17 experimental days in two groups: normally-fed group (open circle, n = 6) and fasted female (normally fed male × fasted female) group (open triangle, n = 6). The fasted female group produced significantly less number of eggs after 3 days of fasting onward. Note that they completely stopped breeding after 1-week-fasting. (B) The mean GSI of normally-fed and 2-week-fasted female (n = 6). 2-week-fasted females showed significantly smaller ovaries. (C) The number of fertilized eggs for 17 experimental days between normally-fed group (open circle, n = 6) and fasted male (fasted male × normally fed female) group (filled triangle, n = 6) showed no significant difference. (D) The mean number of courtships for 17 experimental days between normally-fed group (open circle, n = 6) and fasted male group (filled triangle, n = 6) showed no significant difference. (E) Fasting strongly decreased the body weight in both male and female medaka (each group, n=6). Error bars represent SEM. A, C, D: Mann-Whitney *U* test; $^{**}P < 0.01$, N.S. not significant. B: unpaired two-tailed *t*-test; $^{***}P < 0.001$. E: Bars with different superscript letters are significantly different ($P < 0.001$, Tukey test).

Fig. 2-2. GnRH1 neurons of females, but not of males showed low spontaneous firing activity in the fasting-induced low glucose condition. (A) Representative traces of GnRH1 firing activity of a normally-fed (Fed) female and a 2-week-fasted (Fasted) female recorded in ACSF containing 4.5 mM glucose (4.5 mM G ACSF), and Fasted female recorded in 0.5 mM G ACSF. The mean instantaneous frequency (B), and the number of firings of GnRH1 neurons for 5 min (C) in the following 3 groups: Fed female recorded in 4.5 mM G ACSF (n = 16), Fasted female recorded in 4.5 mM G ACSF (n = 13), and Fasted female recorded in 0.5 mM G ACSF (n = 19). (D) A representative trace of GnRH1 firing activity of Fed male recorded in 4.5 mM G ACSF and Fasted male recorded in 0.5 mM glucose ACSF. (E), (F) The mean instantaneous frequency and the number of firings of GnRH1 neurons for 5 min in the two groups (Fed. male recorded in 4.5 mM G ACSF:

n = 15, Fasted male recorded in 0.5 mM glucose ACSF: n =14). B, C: Steel-Dwass test; *$P < 0.05$, **$P < 0.01$, N.S. not significant. E, F: unpaired two-tailed *t* test; N.S. not significant.

Fig. 2-3. Female specific acute suppression by low-glucose in the firing activity of GnRH1 neurons. (A) A representative whole cell patch clamp recording showing the effect of low-glucose ACSF on firing activity of GnRH1 neurons in a female. (B) A representative whole cell patch clamp recording showing the effect low-glucose ACSF in a female GnRH1 neuron by using a 3 mM glucose-containing intracellular solution. (C), (D) The relative frequency and the relative number of firings of GnRH1 neurons among the three groups: 4.5 mM glucose ACSF, 0.5 mM glucose ACSF, and 0.5 mM glucose ACSF with the 3 mM intracellular glucose (n = 7). (E) A representative trace showing no effect of low-glucose ACSF on the GnRH1 neuronal activity in a male. (F), (G) The relative firing frequency and the relative number of firings of GnRH1 neurons in the two groups: 4.5 mM glucose ACSF, 0.5 mM glucose ACSF (n = 8). C, D: Steel-Dwass test; *$P < 0.05$, N.S. not significant. F, G: Mann-Whitney *U* test; N.S. not significant.

Fig. 2-4. Activators of K_{ATP} channels and AMPK inhibit the GnRH1 firing activity specifically in females. (A-F) In females, the firing activity of GnRH1 neurons was inhibited after application of K_{ATP} channel activator, diazoxide, and a AMP-dependent protein kinase activator, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR). (A) A representative trace of the effect of diazoxide on GnRH1 firing activity in female. Changes in the mean instantaneous frequency (B) and the number of firings (C) in control, 500 μM diazoxide, and wash-out (n = 6). Diazoxide significantly reduced the number of firings, although the effect could not be completely recovered after washout. (D) A representative trace of the effect of AICAR, on GnRH1 neuronal activity in female. Change in the mean instantaneous frequency (E) and the number of firings (F) in control, 2 mM AICAR, and wash-out (n = 6). AICAR significantly decreased the mean instantaneous frequency and the number of firings, whereas the firing activity could not be restored after washout.

(G-L) Unlike those in females, GnRH1 neurons in males did not show significant changes in firing activity after application of diazoxide or AICAR. (G) A representative trace of the effect of diazoxide on firing activity of GnRH1 neurons in male. Change in the mean instantaneous frequency (H) and the number of firings (I) in control, 500 μM diazoxide, and wash-out (n = 8). (J) A representative trace of the effect of AICAR on firing activity of GnRH1 neurons in male. Change in the mean instantaneous frequency (K) and the number of firings (L) in control, 2 mM AICAR, and wash-out (n = 8). Each point connected by a line shows the sequential data obtained in the same cell. (B, C, E, F, H, I, K, L). B, C, H, I, K, L: Steel-Dwass test; $*P < 0.05$, $\#P < 0.1$, N.S. not significant. E, F: Tukey test; $*P < 0.05$, N.S. not significant.

Fig. 2-5. Reduced firing activity by low-glucose solution in female GnRH1 neurons is suggested to be dependent on both K_{ATP} channel activation and AMP-dependent protein kinase pathways. (A-C) Representative traces showing the effects of low-glucose ACSF on female GnRH1 neuronal activity in the presence of tolbutamide (A), dorsomorphin (B), or the both (C). The relative frequency (D) and the relative number of firings of GnRH1 neurons (E) among the eight groups: 4.5 mM glucose ACSF (n = 9), 4.5 mM glucose ACSF with tolbutamide (n = 10), 4.5 mM glucose ACSF with dorsomorphin (n = 6), 4.5 mM glucose ACSF with both tolbutamide and dorsomorphin (n = 8), 0.5mM glucose ACSF (n = 9), 0.5 mM glucose ACSF with tolbutamide (n = 10), 0.5 mM glucose ACSF with dorsomorphin (n = 6), 0.5 mM glucose ACSF with both tolbutamide and dorsomorphin (n = 8).

Supplementary information

Histological analysis of testis

For the histological analysis, I prepared normally-fed and two-week-fasted d-rR male medaka. Histological analysis of the testis was performed, based on the previous report (85). These fish were anesthetized by immersion in 0.02% MS-222, and the testes were rapidly took out and fixed with Bouin's fixative at 4 °C overnight. Then, the testes were dehydrated with methanol. They were routinely processed and embedded in paraffin and sectioned at 8 μm thickness on a microtome. The sections were stained with hematoxylin and eosin (HE). Images were taken with a digital camera (DP70; Olympus, Tokyo, Japan) attached to an upright microscope (BX53-33; Olympus, Tokyo, Japan).

Electrophysiological analysis of glucose-sensing of female GnRH1 neurons under non-reproductive condition

To analyze the glucose-sensing of GnRH1 neurons in female under non-reproductive condition, the pairs of mature male and female *gnrh1:* EGFP transgenic medaka were maintained under a short day condition (10 h light and 14 h dark) at 27°C for 2–6 weeks, because medaka is a long-day breeder. I confirmed their non-reproductive condition by checking that female medaka did not spawn continuously. The whole-cell patch clamp recording was performed in the whole-brain preparation as described before.

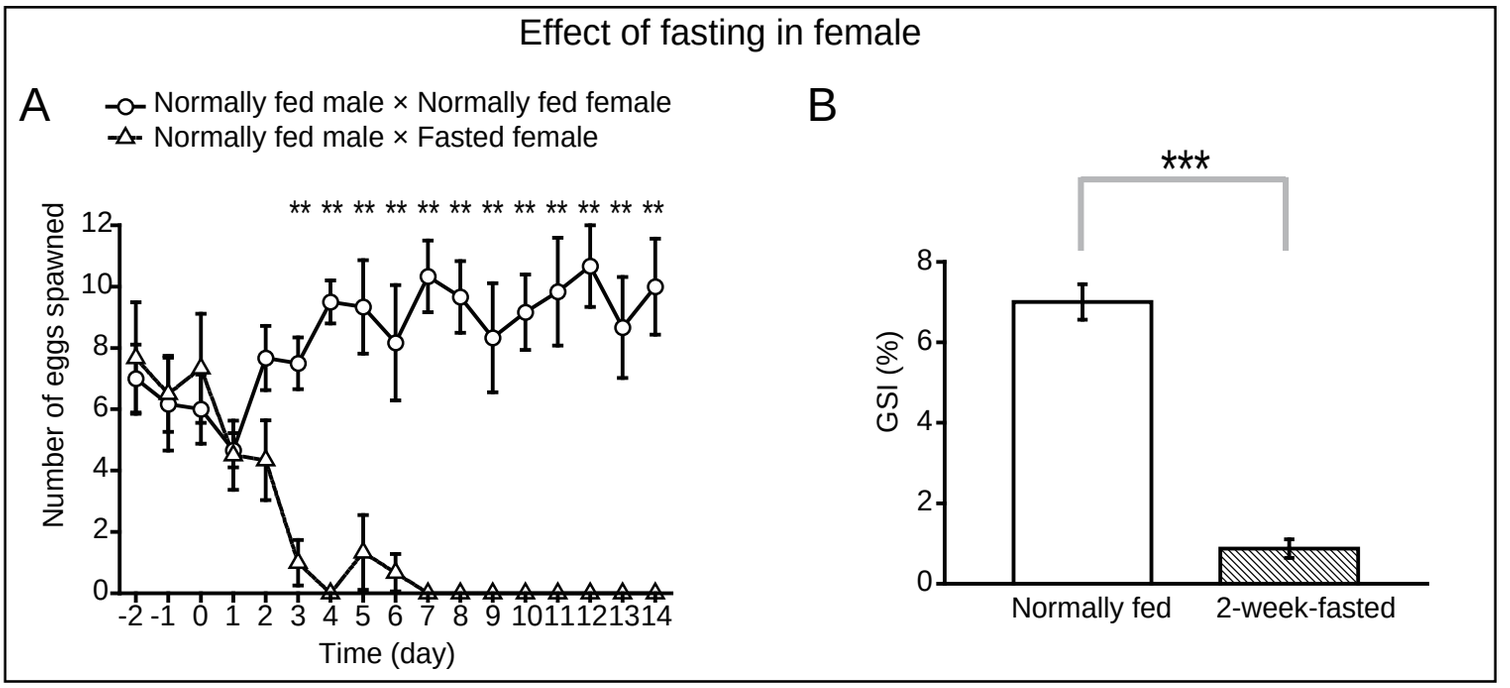
To analyze the effect of low glucose on the firing activity of GnRH1 neuron, ACSF containing 0.5 mM glucose were perfused for 10 min after 10 min-recordings in the standard ACSF containing 4.5 mM glucose. After the perfusion of 0.5 mM glucose ACSF for 10 min, I restored the perfusing solution to 4.5 mM glucose ACSF. The mean frequency or the number of firings in five minutes were calculated between 5 and 10 min after the start of recordings under 4.5 mM glucose ACSF (4.5 mM G before), between 5 and 10 min after the start of perfusion of 0.5 mM glucose ACSF (0.5 mM G), and between 15 to 20 min after the restoring to 4.5 mM glucose ACSF (4.5 mM G after).

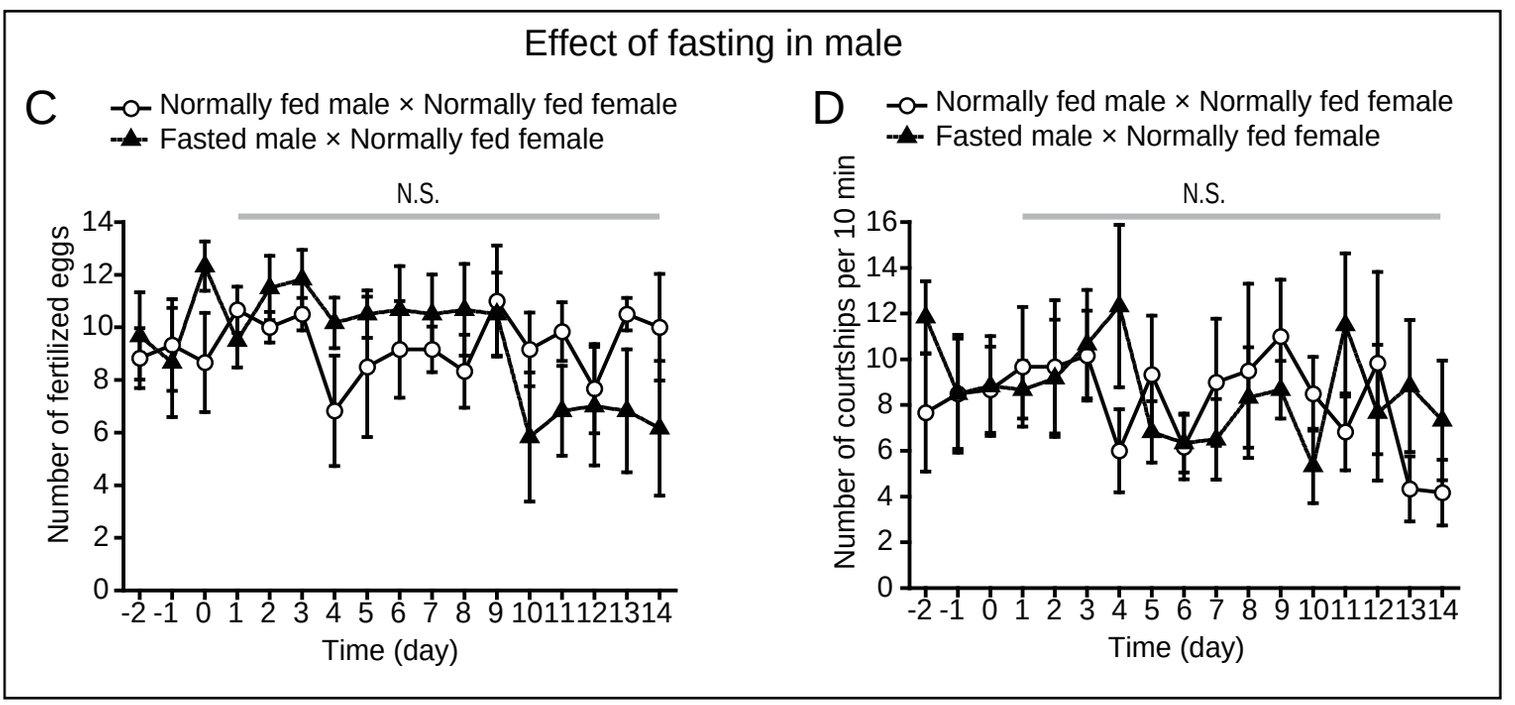
The relative frequency and the relative number of firing were defined as [mean frequency or number of firing (0.5 mM G or 4.5 mM G after) / mean frequency or number of firing (4.5 mM G before)]. Statistical analyses were performed with Kyplot5 software (Kyence, Tokyo, Japan), and significance levels were set as $P < 0.05$.

Supplemental Fig.2-1. Histological analysis of testis of normally-fed and 2-week fasted male medaka. Hematoxylin and eosin (HE)-stained testes of normally-fed (A) and 2-week fasted male medaka (B). SG: spermatogonia, SC: spermatocyte, and SZ: spermatozoa. Scale bar indicates 100 μm.

Supplemental Fig.2-2. Low-glucose induced suppression of firing activity of GnRH1 neurons in female medaka under a non-reproductive condition. (A) A representative whole cell patch clamp recording showing the effect of low-glucose ACSF on firing activity of GnRH1 neurons in female under the non-reproductive condition. (B), (C) The relative frequency and the relative number of firing of GnRH1 neurons among the three groups: 4.5 mM G (before), 0.5 mM G, and 4.5 mM G (after) (n = 5). Steel-Dwass test; $*P < 0.05$, N.S. not significant.

Fig. 2-1


Effect of fasting in female
A
Normally fed male × Normally fed female
Normally fed male × Fasted female
Number of eggs spawned
Time (day)
B
***
GSI (%)
Normally fed
2-week-fasted


Effect of fasting in male
C
Normally fed male × Normally fed female
Fasted male × Normally fed female
N.S.
Number of fertilized eggs
Time (day)
D
Normally fed male × Normally fed female
Fasted male × Normally fed female
N.S.
Number of courtships per 10 min
Time (day)

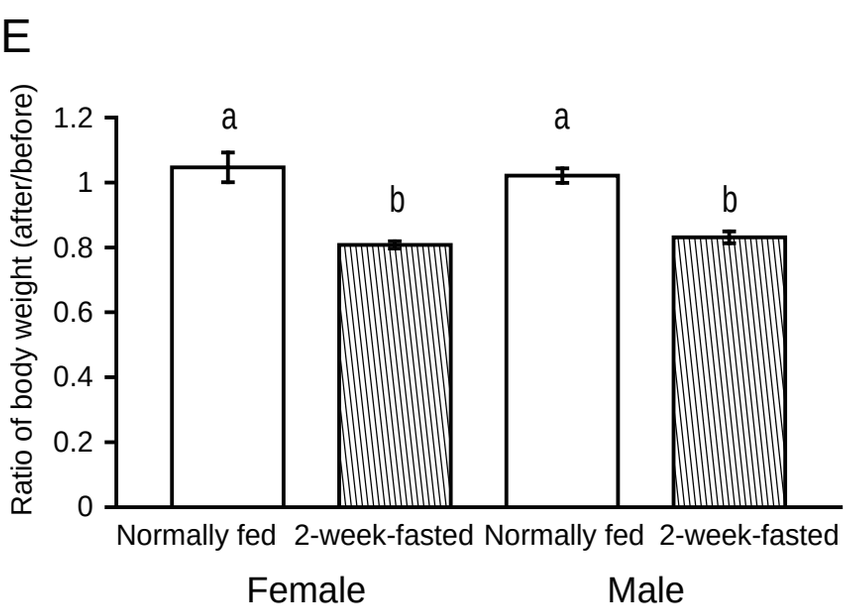

E
Ratio of body weight (after/before)
a
b
a
b
Normally fed
2-week-fasted
Normally fed
2-week-fasted
Female
Male

Fig. 2-2

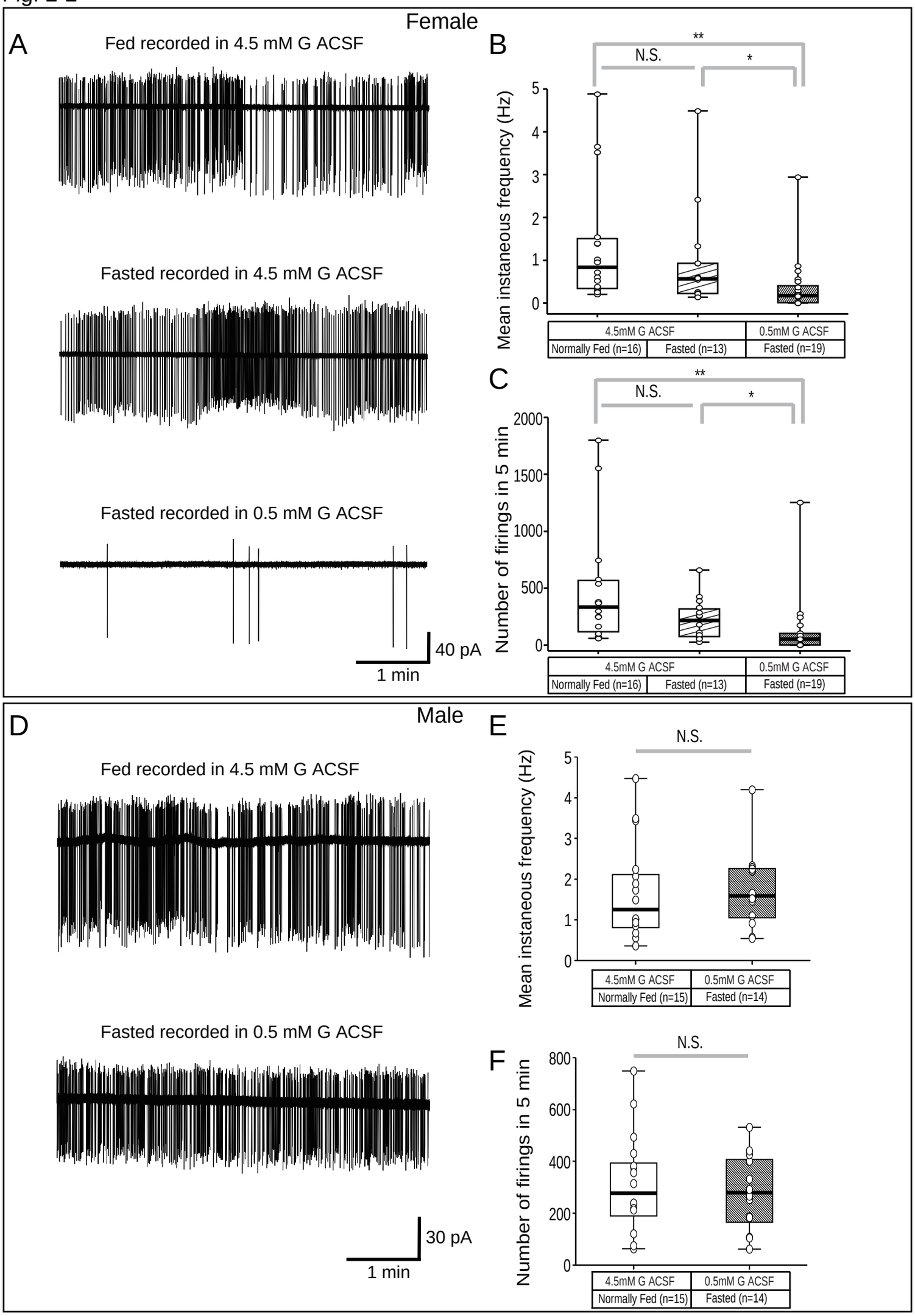
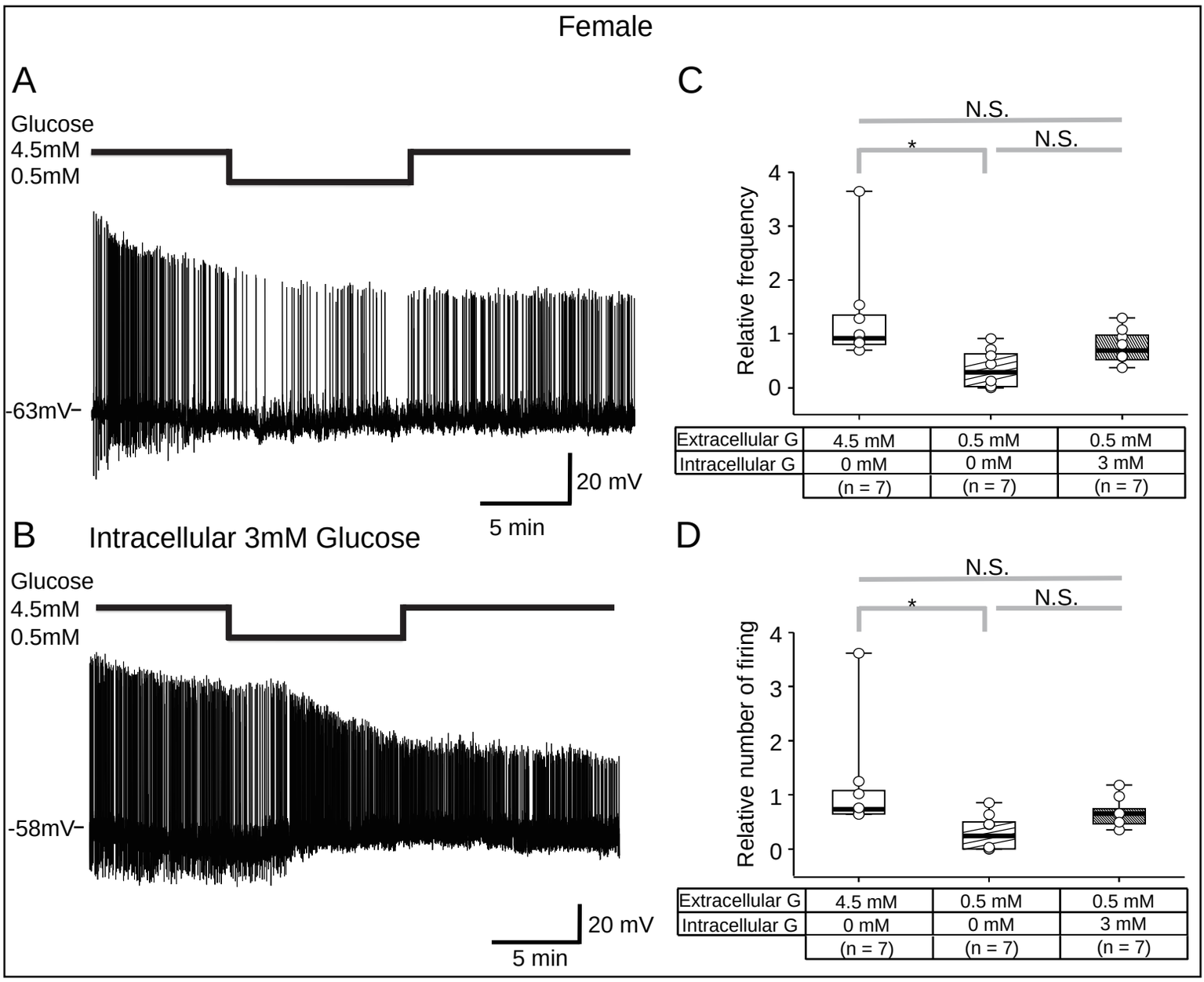

Female
A
Fed recorded in 4.5 mM G ACSF
Fasted recorded in 4.5 mM G ACSF
Fasted recorded in 0.5 mM G ACSF
40 pA
1 min
B
**
N.S.
*
Mean instaneous frequency (Hz)
0
1
2
3
4
5
4.5mM G ACSF
0.5mM G ACSF
Normally Fed (n=16)
Fasted (n=13)
Fasted (n=19)
C
**
N.S.
*
Number of firings in 5 min
0
500
1000
1500
2000
4.5mM G ACSF
0.5mM G ACSF
Normally Fed (n=16)
Fasted (n=13)
Fasted (n=19)
Male
D
Fed recorded in 4.5 mM G ACSF
Fasted recorded in 0.5 mM G ACSF
30 pA
1 min
E
N.S.
Mean instaneous frequency (Hz)
0
1
2
3
4
5
4.5mM G ACSF
0.5mM G ACSF
Normally Fed (n=15)
Fasted (n=14)
F
N.S.
Number of firings in 5 min
0
200
400
600
800
4.5mM G ACSF
0.5mM G ACSF
Normally Fed (n=15)
Fasted (n=14)

Fig. 2-3


Female
A
Glucose
4.5mM
0.5mM
-63mV
20 mV
5 min
B
Intracellular 3mM Glucose
Glucose
4.5mM
0.5mM
-58mV
20 mV
5 min
C
N.S.
*
N.S.
Relative frequency
0
1
2
3
4
Extracellular G | 4.5 mM | 0.5 mM | 0.5 mM
Intracellular G | 0 mM | 0 mM | 3 mM
(n = 7) | (n = 7) | (n = 7)
D
N.S.
*
N.S.
Relative number of firing
0
1
2
3
4
Extracellular G | 4.5 mM | 0.5 mM | 0.5 mM
Intracellular G | 0 mM | 0 mM | 3 mM
(n = 7) | (n = 7) | (n = 7)

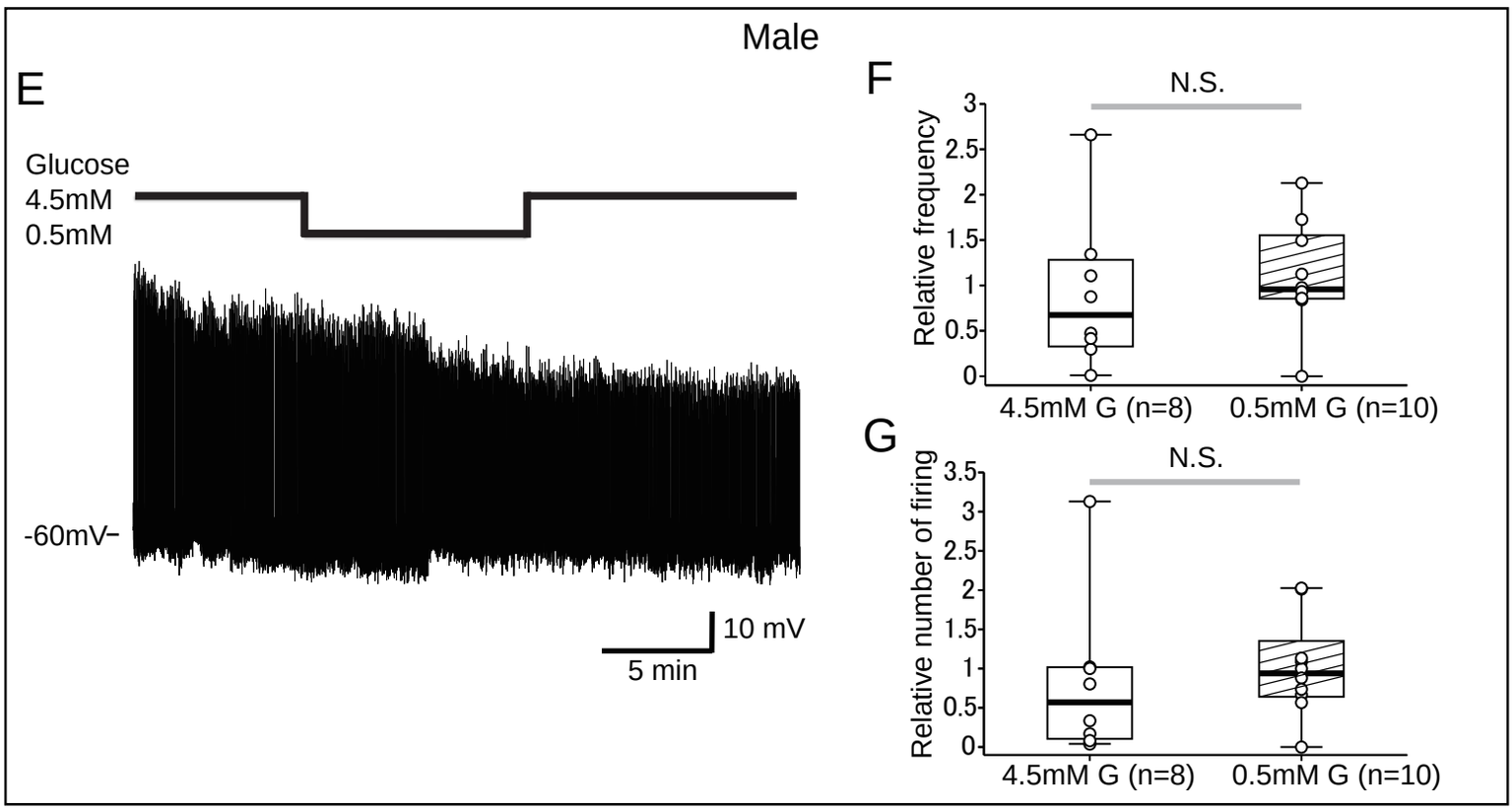
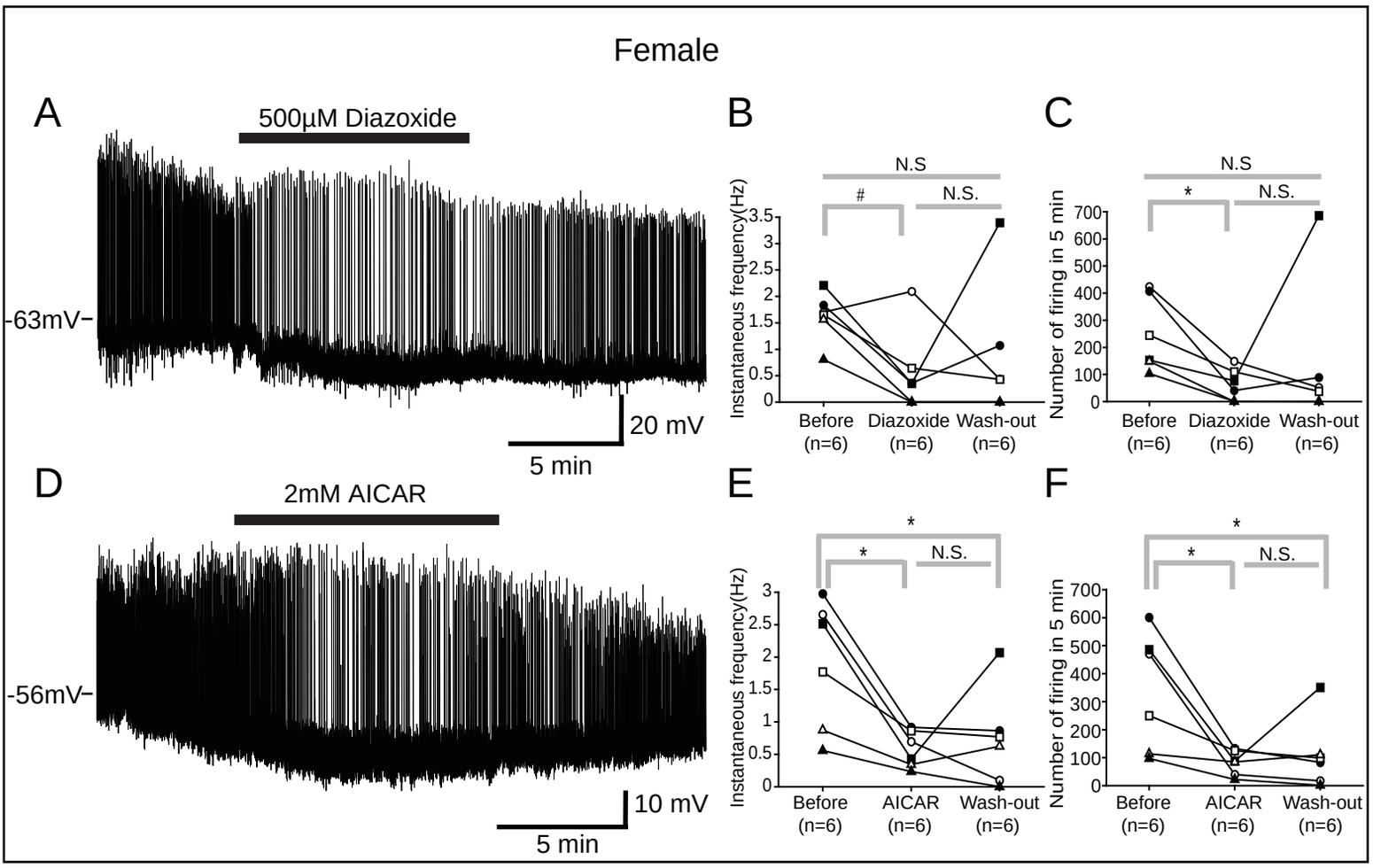

Male
E
Glucose
4.5mM
0.5mM
-60mV
10 mV
5 min
F
N.S.
Relative frequency
0
0.5
1
1.5
2
2.5
3
4.5mM G (n=8)
0.5mM G (n=10)
G
N.S.
Relative number of firing
0
0.5
1
1.5
2
2.5
3
3.5
4.5mM G (n=8)
0.5mM G (n=10)

Fig. 2-4


Female
A
500μM Diazoxide
-63mV
20 mV
5 min
B
N.S
#
N.S.
Instantaneous frequency(Hz)
Before (n=6)
Diazoxide (n=6)
Wash-out (n=6)
C
N.S
*
N.S.
Number of firing in 5 min
Before (n=6)
Diazoxide (n=6)
Wash-out (n=6)
D
2mM AICAR
-56mV
10 mV
5 min
E
*
*
N.S.
Instantaneous frequency(Hz)
Before (n=6)
AICAR (n=6)
Wash-out (n=6)
F
*
*
N.S.
Number of firing in 5 min
Before (n=6)
AICAR (n=6)
Wash-out (n=6)

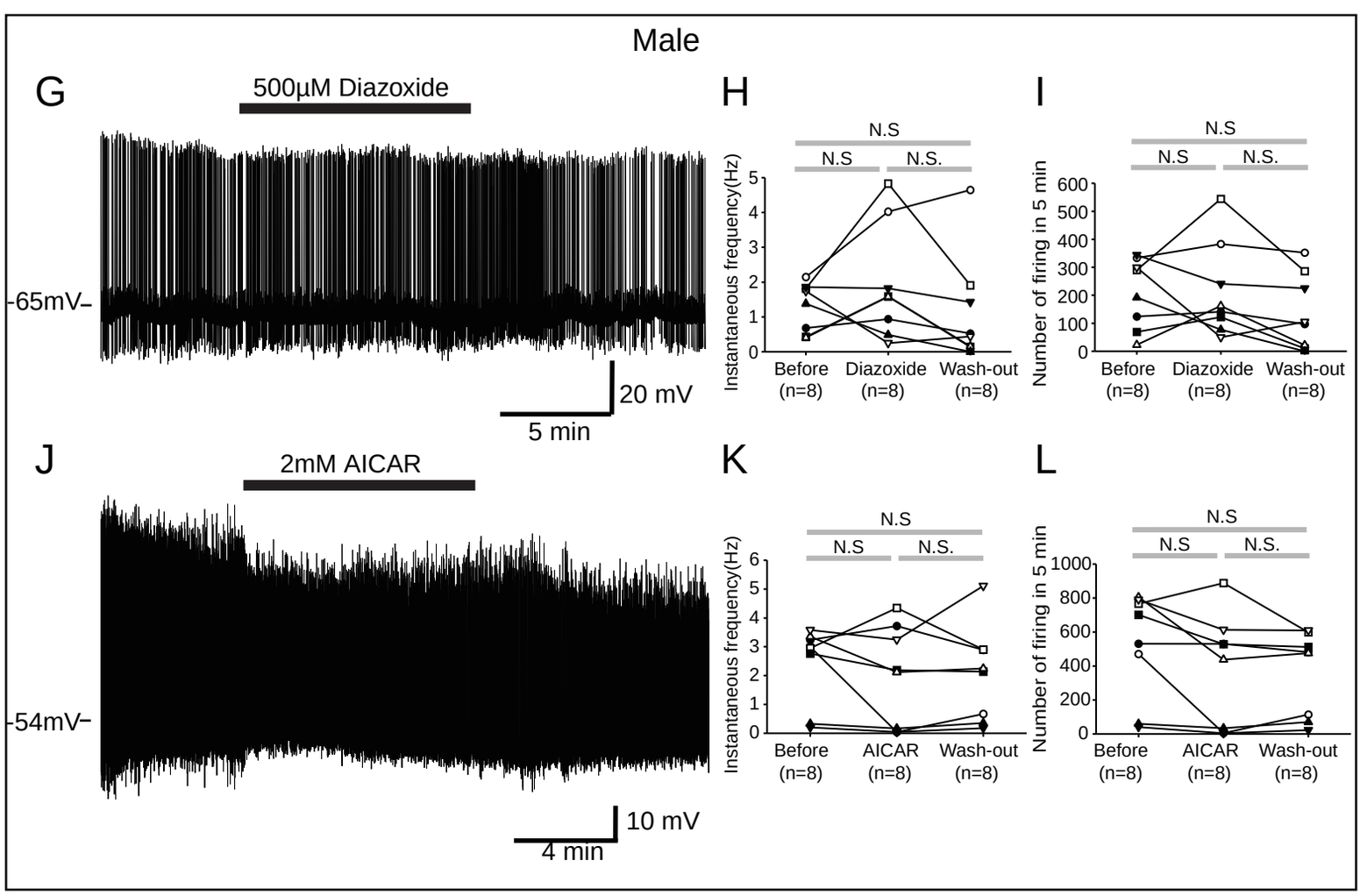
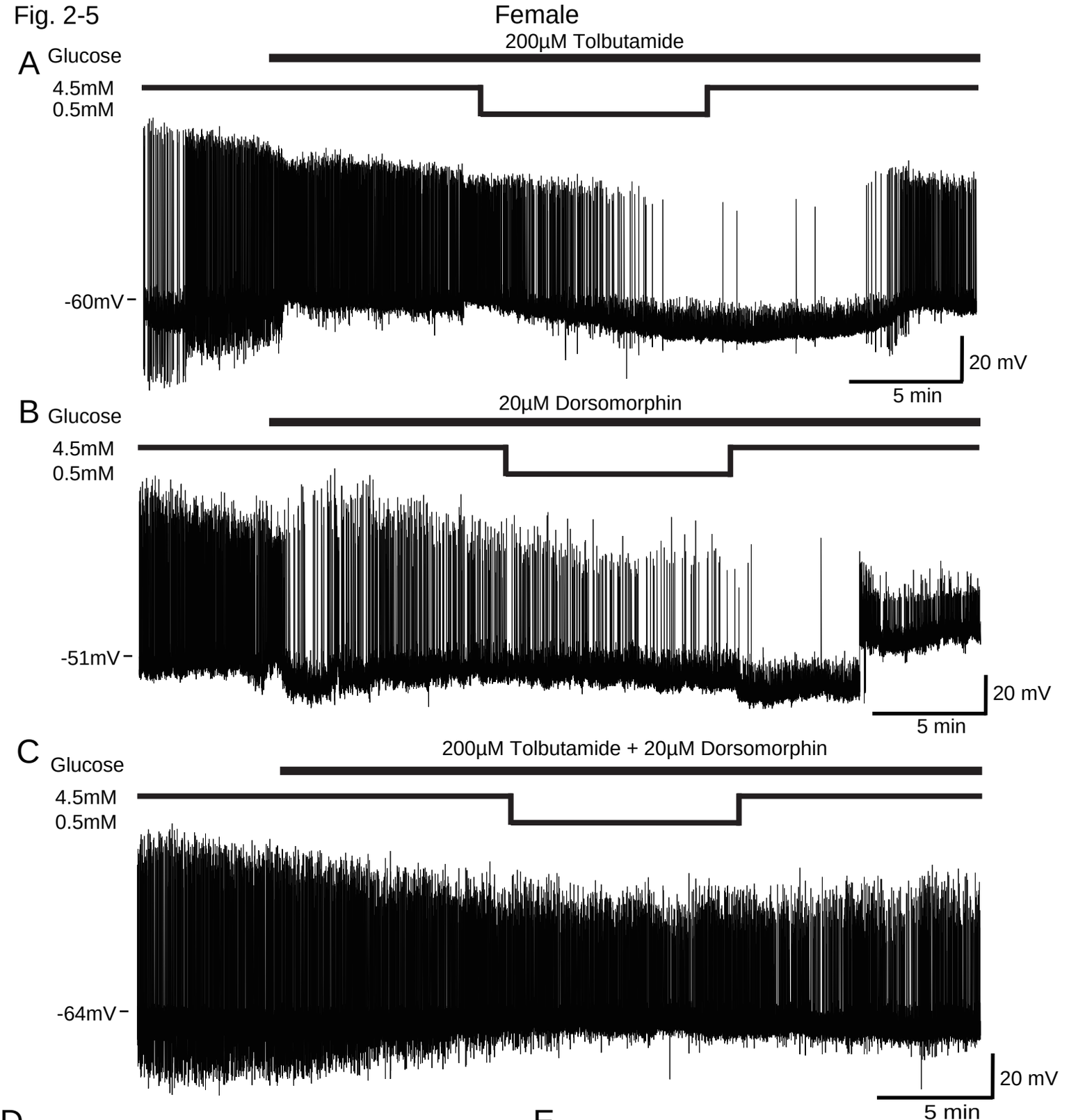
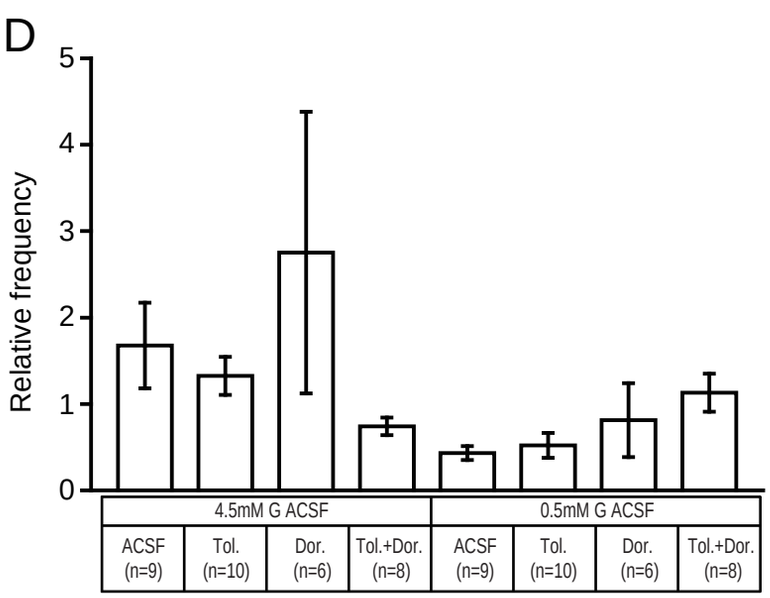
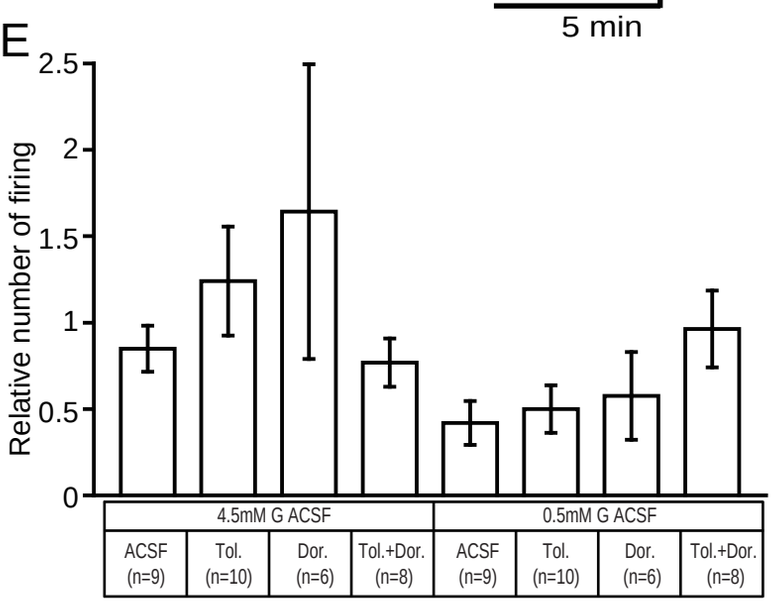

Male
G
500μM Diazoxide
-65mV
20 mV
5 min
H
N.S
N.S
N.S.
Instantaneous frequency(Hz)
Before (n=8)
Diazoxide (n=8)
Wash-out (n=8)
I
N.S
N.S
N.S.
Number of firing in 5 min
Before (n=8)
Diazoxide (n=8)
Wash-out (n=8)
J
2mM AICAR
-54mV
10 mV
4 min
K
N.S
N.S
N.S.
Instantaneous frequency(Hz)
Before (n=8)
AICAR (n=8)
Wash-out (n=8)
L
N.S
N.S
N.S.
Number of firing in 5 min
Before (n=8)
AICAR (n=8)
Wash-out (n=8)

Fig. 2-5

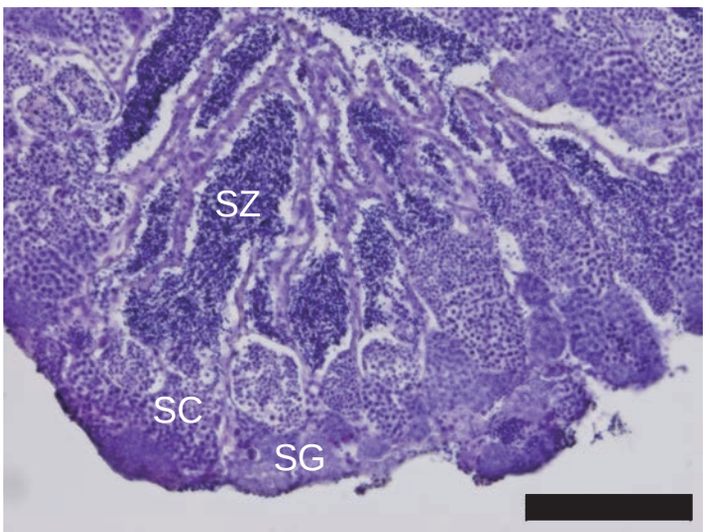

Female
A
Glucose
200μM Tolbutamide
4.5mM
0.5mM
-60mV
20 mV
5 min
B
Glucose
20μM Dorsomorphin
4.5mM
0.5mM
-51mV
20 mV
5 min
C
Glucose
200μM Tolbutamide + 20μM Dorsomorphin
4.5mM
0.5mM
-64mV
20 mV
5 min


D
Relative frequency
0
1
2
3
4
5
4.5mM G ACSF
0.5mM G ACSF
ACSF (n=9)
Tol. (n=10)
Dor. (n=6)
Tol.+Dor. (n=8)
ACSF (n=9)
Tol. (n=10)
Dor. (n=6)
Tol.+Dor. (n=8)

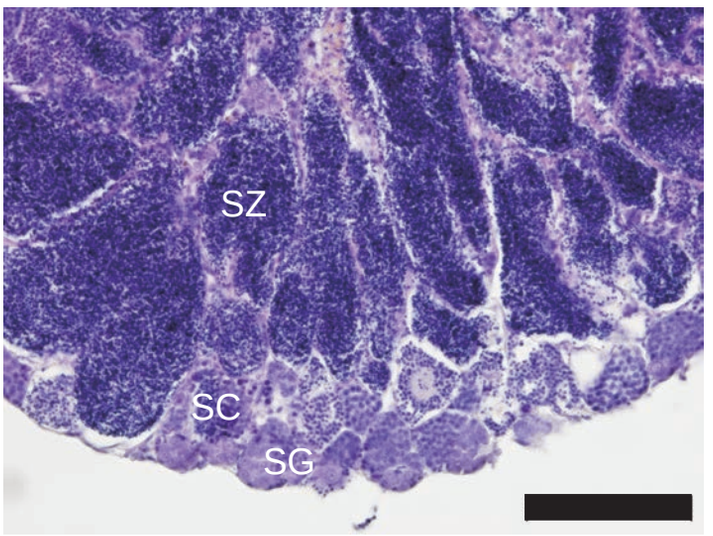

E
Relative number of firing
0
0.5
1
1.5
2
2.5
4.5mM G ACSF
0.5mM G ACSF
ACSF (n=9)
Tol. (n=10)
Dor. (n=6)
Tol.+Dor. (n=8)
ACSF (n=9)
Tol. (n=10)
Dor. (n=6)
Tol.+Dor. (n=8)

Supplemental Fig. 2-1

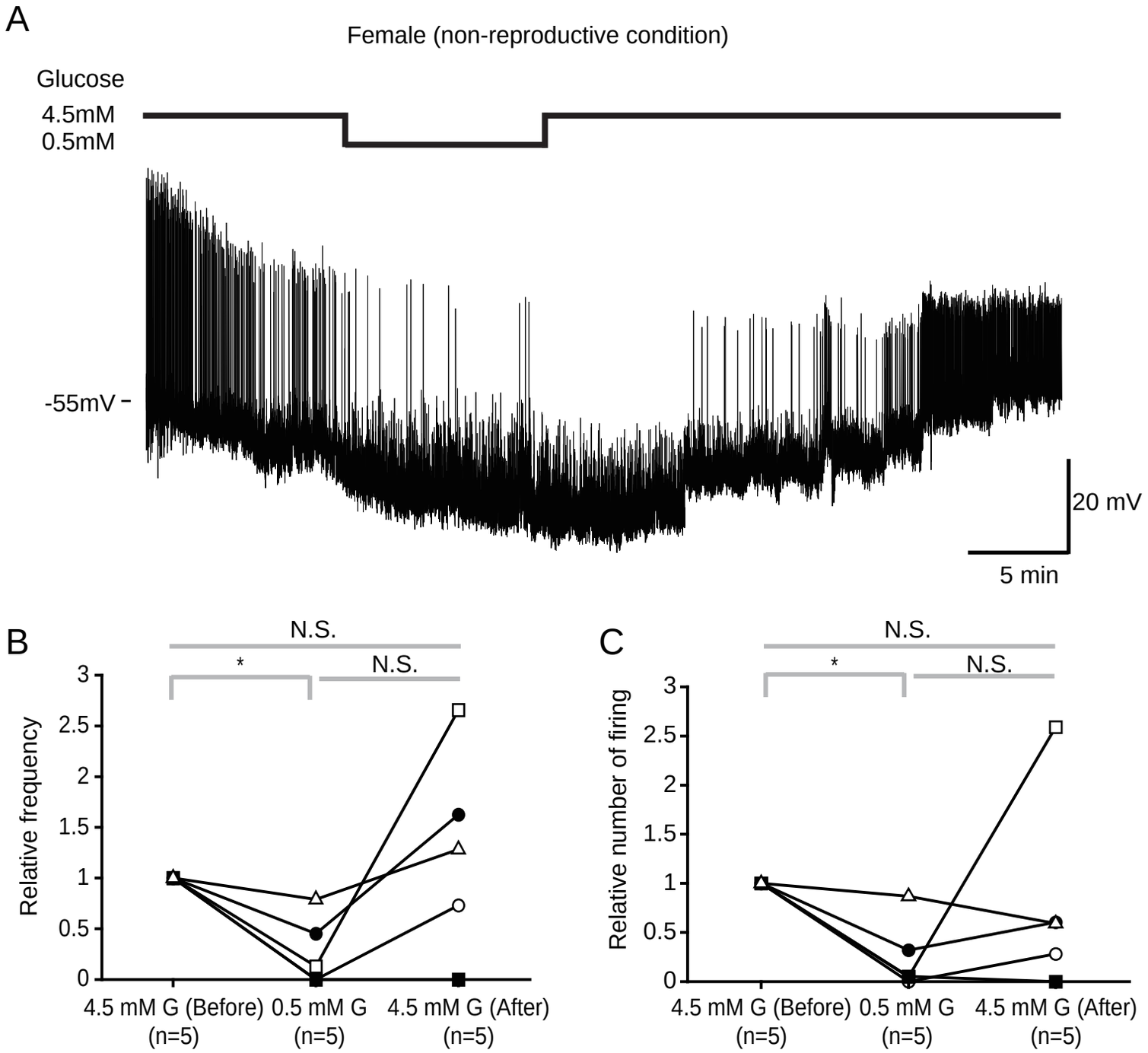
A Testis (Normally-fed male)



B Testis (2-week-fasted male)



Supplemental Fig. 2-2



Chapter 3

本章については、５年以内に雑誌等で刊行予定のため、非公開。

General Discussion

In the present thesis, I aimed at clarifying how animals sense the physiological states and adequately modulate reproduction, by focusing on the hypothalamic peptidergic neurons, kisspeptin and GnRH neurons, which have been reported to be involved in the regulation of reproduction in mammals. In Chapter 1, the physiological analyses showed that steroid-sensitive kisspeptin neurons change their spontaneous neuronal activities in accordance with the breeding state by the change in sex steroids. In Chapter 2, I demonstrated that the spontaneous neuronal activities of GnRH1 neurons show glucose sensitivity only in female medaka, which is correlated to the female-specific suppression of reproduction under the malnutritional state. In conclusion, I demonstrated that the hypothalamic peptidergic neurons, Kiss1 and GnRH1 neurons, sense the physiological states through the modulation of their spontaneous neuronal activities.

Role of steroid-sensitive kisspeptin neurons in the breeding-state dependent modulation of neural/endocrinal systems

Reproductive functions are properly regulated depending on the physiological states. In mammals, kisspeptin neurons have been shown to activate the hypothalamic GnRH neurons directly (36), and are essential for the regulation of reproduction because deletions or mutations of kisspeptin-ligand/receptor gene induce the hypogonadism (28-32). Previous studies suggested that the kisspeptin neurons are involved in linking reproduction and some physiological states, such as nutritional states and breeding states related to seasonality (83,109,110). Therefore, I first analyzed the kisspeptin neurons as the key player of the physiological state-dependent regulation of reproduction. Hypothalamic kisspeptin (Kiss1) neurons in medaka changed their spontaneous neuronal activities according to the breeding state via sex steroids, which suggests that the Kiss1 neurons may act as the sensor of the breeding state (Chapter 1). However, it should be noted that kisspeptin neurons have been recently suggested not to be involved in the reproductive regulation in non-mammalian species. In teleosts, recent morphological studies reported that hypothalamic GnRH1 neurons do not express kisspeptin receptors (25), and knockout analyses also showed that

kisspeptin neurons are not necessary for reproduction (26,27). Besides teleosts, birds have been reported to have lost *kisspeptin* genes during evolution (111-113). These studies suggest that functions of kisspeptin neurons in reproduction are not conserved among vertebrates. Therefore, contrary to my previous assumption, the kisspeptin neurons may not be essential for the regulation of reproduction depending on the physiological states in at least some non-mammalian species including medaka. Although it now turns out that the importance of kisspeptin neurons in reproduction may be limited to only some species such as mammals, the sex steroid-sensitivity of kisspeptin neurons has widely been reported in vertebrates (44). Recent studies reported that kisspeptin is also involved in the regulation of homeostasis and behaviors, apart from reproduction (114-120). In medaka, hypothalamic (NVT) Kiss1 neurons widely project to the brain, such as ventral telencephalon, POA, hypothalamus, and nucleus preopticus pars magnocellularis (47), where kisspeptin receptors are also expressed (25). Previous studies in teleosts reported that telencephalon and POA are involved in the regulation of sexual behaviors (121,122). Additionally, in medaka, kisspeptin receptors are expressed in Vasotocin and Isotocin neurons in nucleus preopticus pars magnocellularis (25), which are suggested to be involved in the regulation of homeostasis and social behaviors. Therefore, the steroid-sensitive kisspeptin neurons may play an important role in modulating these homeostasis/behaviors according to the breeding states (Fig. 4-1). Although the importance of kisspeptin neurons in mammalian reproduction is widely known, the physiological role of kisspeptin neurons in non-mammalian species has been elusive. Future analyses of the functions of steroid-sensitive kisspeptin neurons in non-mammalian species are essential for understanding the diversified physiological role of kisspeptin neurons, which may be the sensor of the breeding condition among vertebrates.

Analyses of the physiological state-dependent regulation of reproduction by focusing on the hypothalamic GnRH neurons

Because the kisspeptin neurons may not be the key to the linkage between reproduction and

physiological states in teleosts, I hypothesized that the information of physiological states is directly transmitted to the hypothalamic GnRH neurons, which are essential for the HPG axis regulation in a wide variety of vertebrate species. The analyses in Chapter 2 demonstrated that GnRH1 neurons in female medaka, but not in male medaka, change their spontaneous neuronal activities according to the extracellular glucose level related to the nutritional state. This sexual difference in glucose-sensing of GnRH1 neurons may be related to the female-specific inhibition of reproduction under malnutritional state in medaka (Fig. 4-1). Interestingly, in contrast to female medaka, GnRH1 neurons in male medaka showed glucose-insensitivity, which was consistent with the fact that males showed no significant effect of malnutritional state on reproduction (Chapter 2). Although the reasons for the sexual differences are unclear, the differences in the cost for reproduction may be related. In various species, energy cost for female oogenesis is suggested to be much higher than that for male spermatogenesis (67,68). Thus, it is hypothesized that the ability of abandoning reproduction under malnutritional states is particularly important for females, because the successful reproduction of females requires higher energy-cost than males. The glucose-sensing of hypothalamic GnRH neurons shown in the present study may be one of the neuronal mechanisms for the female-specific suppression of reproduction under malnutrition. In the present study, the ovary-weight decreased after 2-week-fatsing (Fig. 2-1), which may be related to the suppression of folliculogenesis. Here, it should be noted that hypothalamic GnRH1 neurons in female medaka has been shown to be essential for the control of ovulation by activating pituitary LH cells, but not for the folliculogenesis (85). Therefore, the hypothalamic GnRH1 neurons may not mediate this fasting-induced suppression of folliculogenesis. On the other hand, follicle-stimulating hormone (FSH) has been reported to play an essential role in folliculogenesis (85). Therefore, fasting may have suppressed the release of FSH independent of hypothalamic GnRH1 neurons, which also contributes to the ability of abandoning reproduction in the female medaka under malnutritional state.

Besides the nutritional state, the breeding state changes the release of GnRH peptide in the nerve terminal of GnRH neurons in quails (123). The latest study in medaka suggested that the

reproductive seasonality may be mediated by modulation of responsiveness of LH cells to GnRH peptide (124). Based on the present study and the previous reports, it is suggested that the hypothalamic GnRH neurons play an important role in reproduction depending on the physiological states in the non-mammalian species, in which kisspeptin neurons are not involved in the HPG axis regulation. Future analyses of the relationship between the hypothalamic GnRH neurons and the other physiological states will contribute to the understanding of the significance of GnRH neurons in physiological state-dependent modulation of reproduction.

The role of peptidergic neurons in the physiological state-dependent modulation

Various behaviors and endocrine functions including reproduction are modulated in accordance with the gradual change in the physiological states. Despite such general assumption, it is unclear how the central nervous system, which regulates various behaviors and endocrine functions, is modulated chronically but not acutely according to the physiological states. Peptidergic neurons are suggested to play an important role in modulation of various endocrine functions, homeostatic responses, and behaviors through the release of neuropeptide(s) (125-130). The peptidergic modulation via the release of neuropeptide is suggested to be dependent on the spontaneous neuronal activities, which are endogenously generated without external inputs. In the present thesis, I hypothesized that the spontaneous neuronal activities of peptidergic neurons are regulated in accordance with the physiological states, which contributes to the modulation of behaviors/endocrine functions through the release of neuropeptide. The present analyses suggested that Kiss1 and GnRH1 neurons in female change their spontaneous neuronal activities in accordance with the physiological (breeding/nutritional) states (Fig. 4-1). Remarkably, the percentage of spontaneous bursting Kiss1/GnRH1 neurons was much higher in proper physiological (breeding/well-nutritional) states. The previous studies in some peptidergic neurons reported that a high-frequency (burst) firing promotes the release of neuropeptides (62,131-134). These suggest that the physiological state-dependent modulation of the spontaneous neuronal activities, especially

burst firing activities, in Kiss1/GnRH1 neurons may be reflected to the release of Kiss1/GnRH1 peptides, which contributes to the peptidergic modulation according to the physiological states. This may be one of the neuronal mechanisms for the physiological state-dependent modulation.

At least in some peptidergic neurons, the release of neuropeptide is induced by the high-frequency activity, similar to the hypothalamic GnRH1 and Kiss1 neurons (62,131,132,135). Based on the present analyses and these studies, I propose a working hypothesis that these peptidergic neurons may be involved in the physiological state-dependent modulation of endocrine functions, homeostasis, and behaviors through the control of the high-frequency firing in their spontaneous neuronal activities. The future analyses of the relationships between the spontaneous neuronal activities of peptidergic neurons and the physiological states may contribute to the understanding of the endocrine functions/behaviors according to the physiological states.

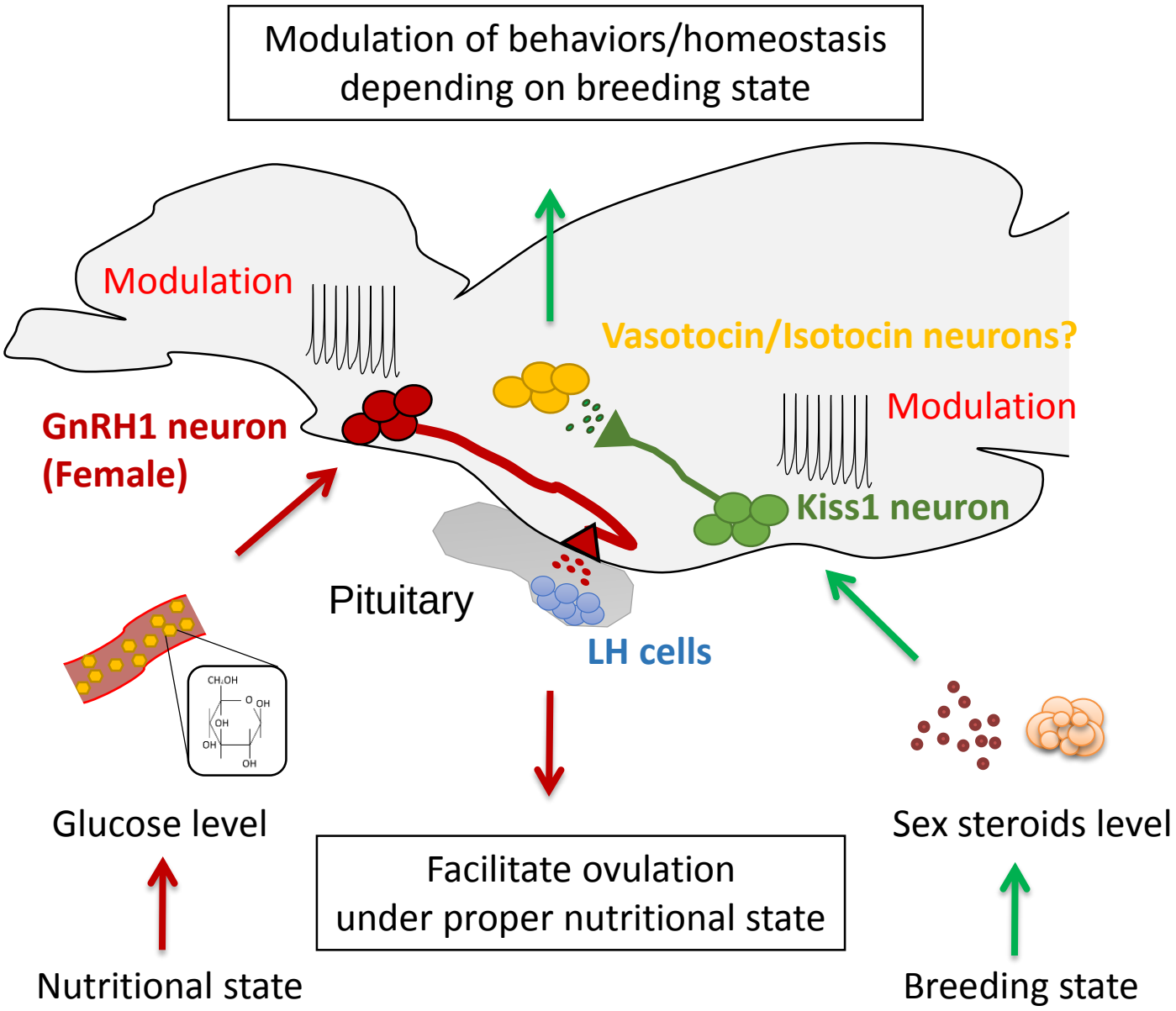
Conclusions

In the present study, I clearly showed neuronal mechanisms for the regulation of reproduction and homeostatic response according to the physiological states, which is mediated by the modulation of the spontaneous neuronal activities in the hypothalamic peptidergic neurons, Kiss1 and GnRH1 neurons. In the present thesis, I used medaka, whose breeding/nutritional states can be easily manipulated without artificial surgery or serious effects on life. Additionally, small and transparent brain of medaka enabled stable recording of the neuronal activity in whole-brain *in vitro* preparations without slicing the brain like in the rodent models. By taking advantage of these features, I first demonstrated that the peptidergic neurons in a condition close to *in vivo* change their spontaneous neuronal activities according to the physiological states. The present thesis not only contributes to the understanding of how animals appropriately facilitate reproduction under appropriate physiological states, but also proposes a model for the physiological state-dependent modulation of the endocrine functions and behaviors.

Figure legend

Fig. 4-1. Schematic illustration of the physiological state-dependent regulation mediated by Kiss1 and GnRH1 neurons in medaka. Spontaneous neuronal activities of the hypothalamic Kiss1 neurons are regulated according to the breeding state via sex-steroids (Chapter 1). A previous study reported that kisspeptin receptors are expressed in Vasotocin and Isotocin neurons, but not in GnRH1 neurons (25). Therefore, the Kiss1 neurons may modulate the Vasotocin and Isotocin neurons and contribute to the breeding state-dependent regulation of behaviors/homeostasis. The hypothalamic GnRH1 neurons in females, but not in males, show glucose-sensitivity of their neuronal activities (Chapter 2). Low glucose induced by malnutritional state inhibits the firing activities of the GnRH1 neurons in females. Such a neural mechanism may contribute to the female-specific suppression of reproduction under malnutrition (Chapter 2).

Fig. 4-1


Modulation of behaviors/homeostasis depending on breeding state
Modulation
Vasotocin/Isotocin neurons?
Modulation
GnRH1 neuron (Female)
Kiss1 neuron
Pituitary
LH cells
Glucose level
Sex steroids level
Facilitate ovulation under proper nutritional state
Nutritional state
Breeding state

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terminal nerve of transgenic medaka (Oryzias latipes). Biology of reproduction 2005; 73:1228-1234

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