学位論文（要約）

Neuroendocrinological studies on the neuronal systems that are regulated by kisspeptin neurons using medaka as a non-mammalian model

（メダカをモデルとしたキスペプチン神経系が制御する機能に関する神経内分泌学的研究）

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Abstract

All biological phenomena are regulated by receiving and integrating various information, cues, and signals from inside and outside the individual for adaptation of its own condition. Sexual reproduction, one of the most important and vital functions of animals, is accomplished by coordinating internal and external signals. Sexual reproduction is composed of gonadal maturation to attain fertility, and the reproductive-state dependent functions such as social behaviors to mate smoothly. For integration and coordination of such functions for successful reproduction, sex steroid signals play pivotal roles. As for gonadal maturation, the hypothalamic-pituitary-gonadal (HPG) axis is essential for the regulation of their reproductive states, which is considered to be common in vertebrates. In mammalian species, a neuropeptide, kisspeptin, which is released by hypothalamic kisspeptin neurons, is a key factor for the HPG axis regulation. On the other hand, various studies have demonstrated that in non-mammalian species, especially in teleosts, involvement of kisspeptin neuronal system in the HPG axis regulation is doubtful. However, the kisspeptin-related genes, which consist of *kiss1*/*2* for the ligands and *gpr54-1*/*2* for the receptors, have been widely conserved in all vertebrates except avian species, suggesting some indispensable functions of kisspeptin neuronal system. Besides, estrogen sensitivity of hypothalamic kisspeptin neurons are also conserved in vertebrates, which is one of the essential properties of reproductive regulation. Therefore, it is strongly suggested that kisspeptin neuronal

system plays some crucial roles in reproduction, but not gonadal maturation itself, although the mechanism largely remains unclear.

In the present study, to discover the unidentified functions of kisspeptin, I used a teleost fish medaka as a non-mammalian model to analyze the neuronal pathways under the regulation of kisspeptin. By taking advantages of medaka such as their daily regular spawning, photoperiod-dependent reproductive cycles, characteristic reproductive behaviors, and easily available genome database for application of genetic tools, I performed multidisciplinary analyses.

In Chapter 1... (5 年以内に雑誌等で刊行予定のため、非公開。)

In Chapter 2, for comprehensive understanding of the neuronal pathways under the regulation of estrogen-sensitive Kiss1 neurons, I examined Gpr54-1 expressing neurons using *gpr54-1*:*EGFP* transgenic medaka. By combination of histological analyses, deep sequencing of the transgenic medaka, and electrophysiological analyses of EGFP-labeled Gpr54-1 expressing neurons, I demonstrate their important properties: their projection sites, neurotransmitters, and neuronal activities. Particularly, Gpr54-1 expressing neurons localized in the ventrolateral preoptic area (vPOA) send their axons robustly to the pituitary, especially proximal pars distalis (PPD) and pars intermedia (PI). Moreover, neuropeptide B (Npb) was identified as a neurotransmitter of vPOA Gpr54-1 expressing neurons, which show direct innervation of the pituitary. Electrophysiological analysis revealed that Kiss1 peptide application facilitates their neuronal activities and probably stimulates their Npb release. Taken together, these lines of

evidence strongly suggest that Gpr54-1/Npb neurons in vPOA play important roles in a reproductive-state-dependent manner. Furthermore, histological analysis of the pituitary suggested that isotocin (IT) and vasotocin (VT) neurons are strong candidates of the targets of vPOA Gpr54-1/Npb neurons, judging from the characteristic structure of their axonal branches in PPD and PI.

Finally, in Chapter 3... (5 年以内に雑誌等で刊行予定のため、非公開。)

(第一章、第三章に関わる記述のみ、5 年以内に雑誌等で刊行予定のため、非公開。)

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(第一章、第三章については、5 年以内に雑誌等で刊行予定のため、非公開。)

Abbreviations

ABC	avidin-biotin complex
ACSF	artificial cerebrospinal fluid
ARC	arcuate nucleus
AS	antisense
AVPV	anteroventral periventricular nucleus
DAB	3, 3’-diaminobenzidine
DIG	digoxigenin
EGFP	enhanced green fluorescent protein
FSH	follicle-stimulating hormone
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
Gpr	G protein-coupled receptor
HPG axis	hypothalamic-pituitary-gonadal axis
i.c.v.	intracerebroventricular administration
IHC	immunohistochemistry
i.p.	intraperitoneal administration
IP	inverse pericam
ir	immunoreactive
ISH	*in situ* hybridization
IT	isotocin
KO	knockout
LH	luteinizing hormone
mdGnRH	medaka GnRH
mdKiss1/2	medaka Kiss1/2 peptide
MS-222	tricaine methanesulfonate
Npb	neuropeptide B
Npw	neuropeptide W

NVT	nucleus ventralis tuberis
OT	oxytocin
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Triton X-100
PI	pars intermedia
PN	pars nervosa
POA	preoptic area
POm	nucleus preopticus pars magnocellularis
POp	nucleus preopticus pars parvocellularis
PPD	proximal pars distalis
RPD	rostral pars distalis
SE	sense
Vd	area ventralis telencephali pars dorsalis
Vp	area ventralis telencephali pars posterior
VP	vasopressin
vPOA	ventrolateral POA
Vs	area ventralis telencephali pars supracommissuralis
VT	vasotocin

(第一章、第三章に関わる略号は、5 年以内に雑誌等で刊行予定のため、非公開。)

General Introduction

(5 年以内に雑誌等で刊行予定の部分の記述については非公開。)

Animals receive and integrate various information and signals from the external and internal environments to adapt to the environments appropriately. Such integration of the signals, which is vital to their survival and breeding, is fulfilled by coordination of the central nervous system and the endocrine system. Regulation of sexual reproduction is one of the most essential functions of animals, which is accomplished by integrated neuroendocrinological regulation. Animals attain sexual maturity and fertility by gonadal maturation, which are dependent on environmental, social, and physiological conditions such as season, social status, and nourishment. In parallel with the gonadal development, sex steroid hormones released from gonads serve as a maturation signal in the brain to activate various functions that are required for successful reproduction, for instance, reproductive behaviors and secondary sexual characteristics to fight or mate with other conspecific individuals of the same or the other sex, respectively. Thus, sexual reproduction is smoothly accomplished by coordination between gonadal maturation and other reproductive-state dependent traits, which is mediated by serum sex-steroid signals, and have been studied as one of the most crucial regulatory mechanisms (1-3).

To date, the so-called hypothalamic-pituitary-gonadal (HPG) axis regulation is considered to be the neuroendocrine system common to almost all vertebrates for the control of their gonadal maturation. The mechanism of the HPG axis regulation has been intensively studied for a long time since the discovery of gonadotropin-releasing hormone (GnRH) early in the 1970's, which is released by GnRH neurons localized in the basal hypothalamic and/or ventrolateral preoptic

area (vPOA) (so called GnRH1 neurons in most animals) (4-6). Previous studies have shown that GnRH neurons are hypophysiotropic and facilitate the release of two types of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary (2,7-11). Here, GnRH neurons release GnRH peptide into the median eminence and GnRH is transported to the pituitary via the portal vessel in mammals, while GnRH1 neurons in teleosts directly project and release GnRH1 peptide to the pituitary. In either way, it is now a general consensus that GnRH neurons form the final common pathway for the HPG axis regulation throughout vertebrates (12-15). However, since the GnRH neurons lack estrogen receptor alpha (ERα), which is essential for steroid feedback regulation of reproduction, the existence of neurons that express ERα and regulate GnRH neurons has been assumed for a long time.

In the early 2000's, a novel neuropeptide, kisspeptin, which is the endogenous ligand of an orphan GPCR, Gpr54, was identified, and there is a growing body of evidence to show that it plays a key role in the HPG axis regulation in mammalian species (2,7-10,16,17). Hypothalamic kisspeptin neurons express ERα and show clear estrogen sensitivity (18-22). In accordance with serum estrogen levels, kisspeptin neurons release kisspeptin and directly regulate GnRH neurons via kisspeptin receptor Gpr54, which is expressed in GnRH neurons. Consistent with this histological and physiological evidence, previous genetic studies demonstrated that the loss of function of *Kiss* or *Gpr54* in mammals including humans causes severe infertility due to hypogonadotropic hypogonadism, or deficiency of gonadal development due to the lack of

expression and release of the gonadotropins (19,23-26). Furthermore, recent comparative studies show that the kisspeptin-related genes, which consist of *kiss1*/*2* for the ligands and *gpr54-1*/*2* for the receptors, and estrogen sensitivity of hypothalamic kisspeptin neurons, are also highly conserved among almost all vertebrates except avian species (27-33). Thus, many studies using non-mammalian species, especially in teleosts, have tried examining possible kisspeptin functions on the HPG axis regulation. However, unlike in mammals, the involvement of kisspeptin in gonadal maturation in non-mammals has been confusing and controversial, partly because of technical limitations, species differences in materials, and methods of the studies. It should be noted, however, that recent histological studies in several different teleost species such as African cichlid (*Astatotilapia burtoni*) (34), European sea bass (*Dicentrarchus labrax*) (35), and medaka (*Oryzias latipes*) (36) have clearly shown that GnRH1 neurons lack expression of *gpr54* mRNA, which is one of the essential properties of GnRH neurons for the kisspeptin regulation of the HPG axis, at least in mammals. Besides, more recent studies have clearly demonstrated that knockout (KO) zebrafish (*Danio rerio*) of kisspeptin-related genes are fertile (37), although some other studies in fish reported that kisspeptin appears to be effective in the HPG axis regulation, although the mechanisms remained unexplained. For example, an older study in Nile tilapia (*Oreochromis niloticus*) used laser-capture microdissection to suggest that GnRH neurons express Gpr54 (38). However, this result is now considered to be caused by the PCR amplification of contaminated Gpr54 mRNAs that are expressed in non-GnRH neurons surrounding the GnRH neurons (see

Kanda et al., 2013 and Grone et al., 2010). Another histological study in chub mackerel (*Scomber japonicus*) reported that *gpr54-1* mRNA expression was localized in GnRH1 neurons using dual labeling *in situ* hybridization (ISH) (39). Besides, previous physiological studies using *in vivo* kisspeptin administration reported in some teleost species that kisspeptin affected gonadotropin expressions, although the mechanism remains still largely unexplained (40-42). One previous study suggested effects of kisspeptin in facilitating LH secretion in goldfish (*Carassius auratus*) (41), although our recent study in goldfish casts doubt on this report, since we could not confirm the results in spite of the same experimental protocol (experimental results included in supplemental data for (43)). Therefore, *in vivo* administration studies using intracerebroventricular (i.c.v.) and intraperitoneal (i.p.) administrations should be treated with caution, since the effects of *in vivo* administration can only be pharmacological effects under unpredictable conditions.

In order to overcome these technical limitations and ambiguities in the previous literature in teleosts as described above, our laboratory has been applying multidisciplinary methods to medaka, a powerful experimental model animal. Medaka is a small fish, which has many features advantageous for the study of reproductive regulation mechanisms. For example, mature medaka show quite regular daily spawning in the morning when they are kept under a long-day photoperiod, mimicking their breeding season. This feature enables us to manipulate the reproductive states of medaka by photoperiod conditions. Moreover, medaka genome database is

available for application of gene-editing tools to generate transgenic or KO lines of fish. By taking advantage of these features of medaka, our analyses have clearly demonstrated that the kisspeptin neuronal system does not affect gonadal maturation by the HPG axis regulation. Previous studies in our laboratory have shown the estrogen sensitivity of hypothalamic Kiss1 neurons (44-46), absence of *gpr54-1/2* mRNA expression in GnRH neurons (36) and fertility of the kisspeptin-related-gene KO medaka (43). Moreover, single-cell physiological analysis including patch clamp recording from neurons in specifically EGFP-labeled transgenic medaka clearly demonstrated that kisspeptin has no effect on the electrical activities of GnRH1 neurons (43). Furthermore, Ca^{2+} imaging of LH cells using a transgenic medaka whose LH cells co-express Ca^{2+} indicator, inverse pericam (IP), also showed that kisspeptin does not stimulate LH cells (43). Taken together, these lines of evidence obtained from multidisciplinary analyses in medaka clearly demonstrate that kisspeptin neuronal system is not involved in any aspect of the HPG axis regulation. Considering the above-mentioned demonstration in distinct teleost species including zebrafish, African cichlid, European sea bass, goldfish, and medaka, the involvement of kisspeptin neuronal system in the HPG axis regulation can be definitely denied in most of the teleosts (Figure 0-1).

On the other hand, the existence of highly conserved kisspeptin-related genes and estrogen sensitivity of kisspeptin neurons in vertebrates except birds, and prosperity of birds without kisspeptin strongly suggest the existence of some important functions of kisspeptin neuronal system other than the facilitation of gonadal maturation via the HPG axis regulation. However,

the mechanisms of such regulation still remain enigmatic. Considering the fact that estrogen sensitivity is also present in most of the non-mammalian species including medaka (44-46), I surmised that Kiss1 neuronal system may play an important role in the regulation of reproductive functions that lead to a successful reproduction via pathways other than the HPG axis regulation itself. Therefore, I performed comprehensive analyses to search for thus far unrevealed functions of kisspeptin neuronal system by taking advantage of medaka as mentioned above.

In Chapter 1… (5 年以内に雑誌等で刊行予定のため、非公開。)

In Chapter 2, I aimed at finding the other neuroendocrine/behavioral regulatory pathways of Kiss1-Gpr54-1 neuronal system. To analyze the functions of the Kiss1 receptor (*gpr54-1*)-expressing neurons, I used a transgenic line of medaka whose *gpr54-1* expressing cells are specifically labeled by EGFP. Using this genetically modified medaka, I performed multidisciplinary analyses to elucidate functions of the steroid-sensitive kisspeptin neural system, which may be conserved in vertebrates.

First, by histological analyses, I analyzed the distribution of Gpr54-1 expressing neurons. Together with the analysis of their axonal projection areas, I performed a combination of deep sequencing and dual labeling of histological analysis to identify neurotransmitters of Gpr54-1 expressing neurons. From these analyses, I newly found relationships between Kiss1-Gpr54-1 neuronal system and neuropeptide B (Npb) neuronal system. Npb was identified in the early 2000's as a novel neuropeptide (47-50). Particularly, I found a group of Npb neurons in the

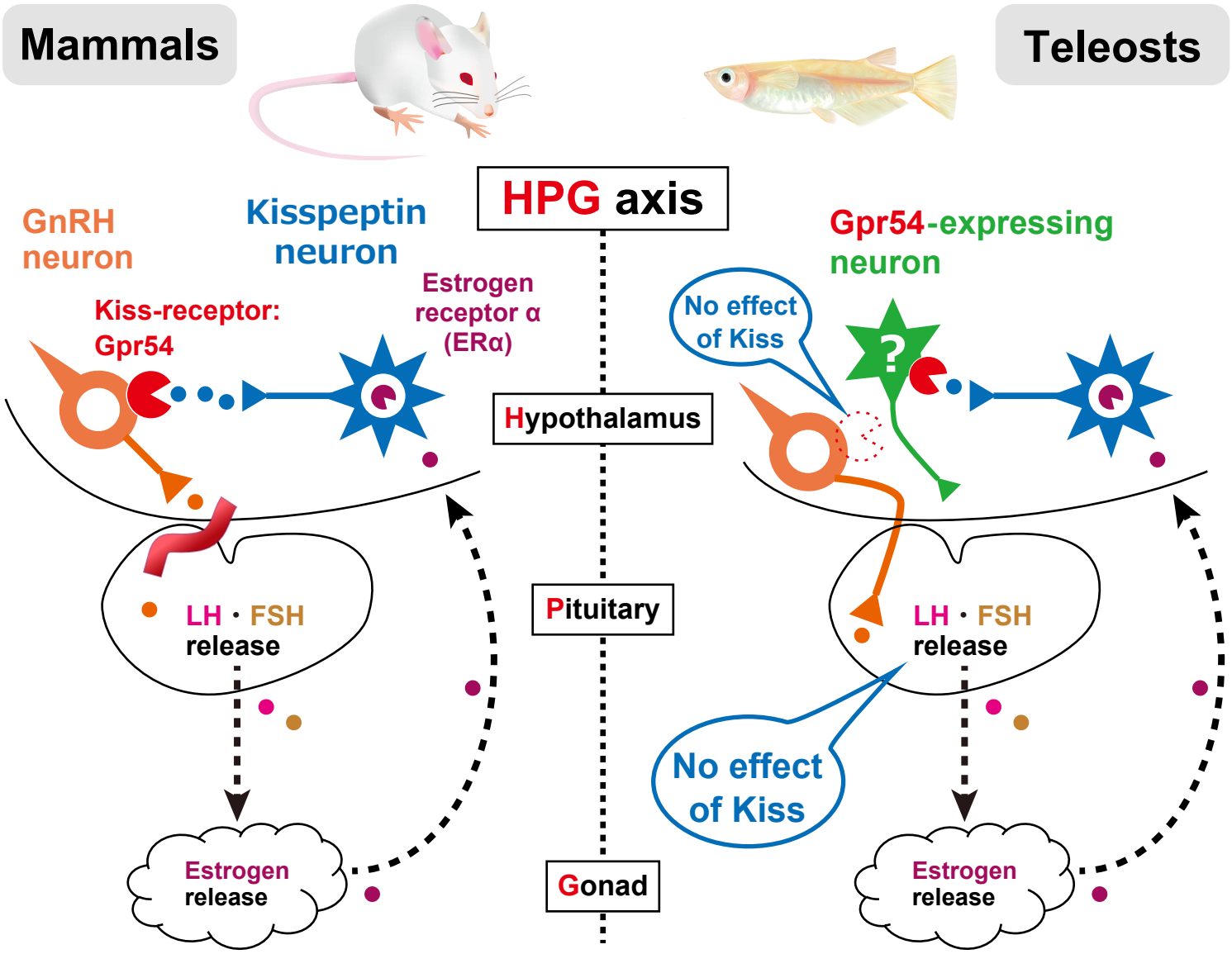
ventrolateral preoptic area (vPOA) co-expressing Gpr54-1 (Gpr54-1/Npb neurons in vPOA) and having robust axonal projections to the pituitary, suggesting an Npb regulation of some pituitary hormones. Next, electrophysiological analysis of Gpr54-1/Npb neurons in vPOA was performed to reveal their spontaneous neuronal activities and response to Kiss1 peptide signals. The results suggested that Kiss1 peptide facilitated spontaneous activity of Gpr54-1/Npb neurons in vPOA, which probably leads to Npb release in the pituitary. Moreover, during morphological search for the targets of Gpr54-1/Npb neurons in vPOA, I found a characteristic anatomical relationship between the axon fibers of Gpr54-1/Npb neurons in vPOA and those of isotocin (IT)/vasotocin (VT) neurons. The axonal branches of the two types of neurons were intertwined with each other in the proximal pars distalis (PPD) and the pars intermedia (PI) of the pituitary. Therefore, the IT/VT neurons are suggested to be strong candidates for Npb targets. IT/VT peptides, the orthologous products of mammalian oxytocin and vasopressin, respectively, have also been widely conserved in vertebrates, and they are suggested to be involved in various neuroendocrine functions including osmoregulation, stress response, and social behaviors (51-68). Here, I discovered novel neuronal pathways of Kiss1-Gpr54-1 system, which probably regulate IT/VT neurons via Npb signaling.

In Chapter 3… (5 年以内に雑誌等で刊行予定のため、非公開。)

Figure 0-1 legend

Schematic illustrations for the hypothalamic-pituitary-gonadal (HPG) axis regulation and kisspeptin neuronal system in mammals and teleosts. In mammalian species (left), it has been generally accepted that hypothalamic kisspeptin (Kiss1) neurons (blue), which possess estrogen sensitivity to receive gonadal estrogen signals by co-expression of estrogen receptor α (ERα) (magenta), directly stimulate GnRH neurons (orange) via a kisspeptin receptor, Gpr54 (red). In turn, GnRH neurons stimulate the release of two types of gonadotropins, LH and FSH in the pituitary (pink and gold). On the other hand, in teleost species (right), the kisspeptin neuronal system is neither involved in the regulation of hypophysiotropic GnRH neurons nor gonadotrophs. Nevertheless, hypothalamic estrogen-sensitive Kiss1 neurons are common between mammals and teleosts, suggesting some important common roles of Gpr54-expressing neurons. Here, to find the significance of Kiss1 neural system in non-mammals, I analyzed the Kiss1-Gpr54-1 system in medaka by various methods.

Figure 0-1


Mammals
Teleosts
HPG axis
GnRH neuron
Kisspeptin neuron
Kiss-receptor: Gpr54
Estrogen receptor α (ERα)
Hypothalamus
Pituitary
Gonad
LH・FSH release
Estrogen release
Gpr54-expressing neuron
No effect of Kiss
?
No effect of Kiss
LH・FSH release
Estrogen release

Chapter 1

(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。)

Chapter 2

Multidisciplinary Analyses of The Neuronal Pathways of Gpr54-1-expressing Neurons Reveal Various Non-GnRH Neuropeptide Regulation in Medaka

(5 年以内に雑誌等で刊行予定の部分の記述については非公開。)

Abstract

Kisspeptin neuronal system, which consists of a neuropeptide kisspeptin and its receptor Gpr54, is considered in mammals to be a key factor of reproductive regulation, so called hypothalamic-pituitary-gonadal (HPG) axis. However, in non-mammalian vertebrates, especially in teleosts, existence of kisspeptin regulation on the HPG axis is doubtful. Here, I applied multidisciplinary techniques to a teleost fish, medaka and examined possible kisspeptin regulation other than the HPG axis. As kisspeptin genes are completely conserved among vertebrates except birds, I surmised that kisspeptin should have some important non-reproductive functions in vertebrates. Therefore, to discover novel functions of kisspeptin, I generated a *gpr54-1*:*EGFP* transgenic medaka, whose *gpr54-1* expressing cells are specifically labeled by EGFP. Analysis of neuronal projection of *gpr54-1*:*EGFP* expressing neurons showed that these neurons in ventrolateral preoptic area (vPOA) project to the pituitary and are probably involved in endocrine regulation other than gonadotropin release. Furthermore, combination of deep sequencing, histological and electrophysiological analyses revealed various novel neural systems that are under control of kisspeptin neurons: those expressing neuropeptide Yb, cholecystokinin, isotocin, vasotocin, and neuropeptide B. Thus, my new strategy to genetically label receptor-expressing neurons gives insights into various kisspeptin-dependent neuronal systems that may be conserved in vertebrates.

Introduction

Sexual reproduction, one of the most essential characteristics of animals, cannot be accomplished solely by gonadal maturation. During the breeding season, successful reproduction requires various behaviors such as aggression to reject other individuals, as well as secondary sex characteristics such as nuptial coloration or pheromones to attract conspecific mates of the other sex. These characteristics specific to reproductive season are considered to be regulated by sex steroid signals (1-3). In the brain, the sex steroid-sensitive neurons have been suggested to play critical roles in both behavioral and hormonal regulation. Regarding gonadal maturation, the hypothalamic-pituitary-gonadal (HPG) axis plays a crucial role in vertebrates (2,9,10). In mammalian species, a recently found peptidergic neural system, kisspeptin neuronal system, is considered to play an essential role in the HPG axis regulation. The hypothalamic kisspeptin neurons, which release kisspeptin in accordance with serum sex steroid-concentration, directly stimulate GnRH (Gonadotropin-releasing hormone) neurons, which express a kisspeptin receptor, Gpr54 (7). The GnRH release, in turn, stimulates the release of gonadotropins (Luteinizing hormone: LH and Follicle-stimulating hormone: FSH) from pituitary and promotes gonadal maturation (2,7,9,10). Consistent with this, the lack of functional *Kiss* or *Gpr54* in mammals including humans causes infertility due to hypogonadotropic hypogonadism (23-26). Essential components of reproduction are considered to be conserved widely in vertebrates. In fact, GnRH, LH, and FSH are proven to be essential for reproduction in both mammals and teleosts (12-15).

At present, there is a general consensus that hypothalamic kisspeptin neuron is sensitive to estrogen, which is one of their essential properties to be a mediator of sex steroid-dependent regulation of reproduction in both teleosts (44-46,69) and mammals (18-22) and that mammalian kisspeptin neuronal system plays a crucial roles in the HPG axis regulation. However, as for the kisspeptin neuronal system in non-mammalian species, several recent different approaches demonstrated the absence of kisspeptin involvement in reproductive regulation in several different species, and the reproductive function of kisspeptin is now doubtful.

On the other hand, kisspeptin-related genes themselves, *kiss1* and *kiss2* for ligands and *gpr54-1* and *gpr54-2* for their receptors, are widely conserved in all vertebrates except birds (27,29,33,69). Among these paralogous genes, *kiss2* and *gpr54-2* have been conserved in most teleost species, while they have been lost in mammals after their divergence from monotremes during evolution (27,29,33,69).

In the present study, I aimed to find the other neuroendocrine/behavioral regulation of Kiss1 by using a teleost species, medaka (*Oryzias latipes*). As the previous studies have shown that hypothalamic Kiss1 neurons are the only steroid-sensitive kisspeptin neurons in medaka (44-46), I analyzed the neuronal pathways and their functions, which are supposed to be mediated by reproductive-state-dependent Kiss1 signaling. Medaka is one of the best non-mammalian experimental models because of the ease of access to genetic manipulation, their photoperiod-dependent reproductive cycles, and small and transparent brains for physiological analyses. To

analyze the Kiss1 receptor (*gpr54-1*)-expressing neurons, I established a transgenic line whose *gpr54-1* expressing cells are specifically labeled by EGFP. By using these genetically modified medaka lines, I performed multidisciplinary analyses to elucidate functions of the steroid-sensitive kisspeptin neural system, which is conserved in vertebrates.

Materials and methods

Animals

Male and female wild type d-rR medaka (*Oryzias latipes*), the transgenic medaka (*gpr54-1*:*EGFP*) were used in this study. All of them were maintained in pairs or shoals under a 14 h light, 10 h dark photoperiod condition at a water temperature of 27 °C for their breeding condition. Fish were fed two to four times a day with live brine shrimp and/or commercial flake food (Medaka-bijin, Spectrum brands, Yokohama, Japan) until they were used for experiments. All the fish maintenance and the experiments were conducted in accordance with the protocols approved by Committee on Animal Care and Use of the Graduate School of Science, the University of Tokyo (permission number, 15–3).

Generation of a *gpr54-1*:*EGFP* transgenic medaka

The medaka genomic DNA fragment containing the 5'-flanking region (3 kb) of *gpr54-1* was amplified by PCR, using the sense and antisense primer pair (Table 2-1) and fused with an *EGFP*

open reading frame fragment by overlap extension PCR using PrimeSTAR (Takara Bio, Shiga, Japan). Because the expression level of *gpr54-1* during the early developmental stages of medaka was suggested to be low, I prepared a double-promoter construct for effective screening, by adding zebrafish (*Danio rerio*) cardiac myosin light chain 2 (cMLC2) enhancer (44). Next, this whole fragment (approximately 6 kb) (Figure 2-1A) was cloned into the pCR-TOPO-XL cloning vector (Invitrogen, Carlsbad, CA). I established the transgenic line that possesses the transgene in the genome by microinjection and following screening processes as described previously (70). By this strategy, I successfully observed EGFP-positive cells in the adult brains (Figure 2-1B). As for the *lhb*:*IP*, *gnrh1*:*EGFP*, and *lhb*:*IP*;*gnrh1*:*EGFP* transgenic medaka, I used the same lineages reported in the previous studies in our laboratory (11,70,71).

Specificity of EGFP labeling on *gpr54-1* expressing cells

In order to confirm the specificity of EGFP expression in *gpr54-1* expressing cells, I performed dual labeling of EGFP immunohistochemistry (IHC) and *gpr54-1* mRNA *in situ* hybridization (ISH) on frozen brain sections of this transgenic medaka. A *gpr54-1*-specific digoxigenin (DIG)-labeled mRNA probe was prepared (Table 2-1) and applied to IHC and ISH procedures, following a standard protocol that I documented previously (11,44,45) with some minor modifications. Briefly, adult male and female medaka were anesthetized by immersion in 0.02% tricaine methanesulfonate (MS-222) (Sigma-Aldrich, Darmstadt, Germany) and quickly decapitated or

fixed by perfusion with 4% paraformaldehyde (Nacalai Tesque, Japan) in PBS (Takara). The whole brains were quickly dissected out and fixed with 4% paraformaldehyde in PBS for 2-6 hours (without perfusion fixation) or 10-15 min (after perfusion fixation), and then substituted with 30 % (w/v) sucrose (Wako, Osaka, Japan) in PBS overnight. Frontal sections (20-30 μm) were prepared by using a cryostat (CM 3050S; Leica Microsystems, Wetzlar, Germany), and mounted onto MAS-GP typeA-coated glass slides (Matsunami, Osaka, Japan). To detect EGFP-immunoreactive (EGFP-ir) cells, I used anti-EGFP antibody raised in rabbit (72) (1:1000; a generous gift from Dr. Kaneko and Dr. Hioki, Kyoto University, Kyoto, Japan, or Thermo Fisher Scientific, A-11122 (Table 2-2)). To detect *gpr54-1* mRNA, the sections were hybridized with 100-200 ng/ml DIG-labeled antisense cRNA probes prepared from the medaka-brain cDNA samples using a labeling kit (Roche Applied Science, Mannheim, Germany) overnight at 58°C. A sense cRNA probe was used as negative controls. For dual-fluorescent visualization steps of IHC and ISH signals, AlexaFluor488 conjugated anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA) and HNPP/FastRed (HNPP Fluorescent Detection Set);(Roche Applied Science) were applied respectively. The sections were observed by a DM5000 B fluorescence microscope (Leica) and a LSM-710 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) for the examination of co-localization for *gpr54-1* mRNA and EGFP.

Histological analysis of projection sites of *gpr54-1* expressing cells

To analyze projection sites of *gpr54-1* expressing cells, I used the *gpr54-1*:*EGFP* transgenic medaka and performed single EGFP IHC with 3, 3'-diaminobenzidine (DAB) (Wako) for chromogenic labeling, following the IHC protocol described above. The chromogenic signals were observed using a DM 5000 B microscope (Leica Microsystems, Wetzlar, Germany). I also performed whole-mount IHC with fluorescent labeling in the same protocol followed by tissue clarification as previously reported (73). The transparent samples were observed using a Lightsheet Z.1 microscope (Carl Zeiss). Regarding dual labeling of Gpr54-1 expressing cells and isotocin (IT) or vasotocin (VT) neurons, I used anti-EGFP antibody raised in rat (1:1,000; GF090R, Nacalai Tesque, Kyoto, Japan) and anti-IT or VT antibodies raised in rabbit (1:1,000; generous gift from Dr. S. Kawashima, Zenyaku Kogyo Co.Ltd., Tokyo, Japan (Table 2-2)), respectively. EGFP immunoreactivities were visualized with Alexa Fluor 488-conjugated streptavidin (1:500; Invitrogen) after signal-amplification with ABC Elite kit (Vector Laboratories, Burlingame, CA) and IT or VT immunoreactivities were visualized with AlexaFluor555 conjugated anti-rabbit IgG (1:800; Invitrogen). The projection sites were based only on the detected fibers, which originated from validated nuclei. For the nomenclature of the medaka brain nuclei, I followed the Medaka Histological Atlas (Wakamatsu et al., Medaka Histological Atlas, edited by the Editorial Board of Medaka Histological Atlas of National BioResource Project Medaka, http:// www.shigen.nig.ac.jp/medaka/medaka_atlas).

Deep sequencing of *gpr54-1*:*EGFP* expressing cells

The whole brains of adult male and female medaka were prepared as described above and placed in silicone-bottom dish filled with an artificial cerebrospinal fluid (ACSF) consisting of (in mM) 134 NaCl, 2.9 KCl, 1.2 $MgCl_2$, 2.1 $CaCl_2$, 10 HEPES, and 15 glucose (pH 7.4, adjusted with NaOH). Whole-brain *in vitro* preparations were carefully peeled off the meninges covering the telencephalon and hypothalamus in ACSF. Next, I identified *gpr54-1* expressing cells by their EGFP fluorescence under an upright fluorescent microscope with infrared-differential interference contrast optics (Eclipse E-600FN; Nikon, Tokyo, Japan) and an infrared charge-coupled device camera (C3077–78; Hamamatsu Photonics, Shizuoka, Japan). Then I collected five EGFP-positive cells in ventrolateral preoptic area (vPOA) by suction of the pipettes made from borosilicate glass capillaries of 1.5 mm outer diameter (GD-1.5; Narishige, Tokyo, Japan). The pipettes were pulled using a Flaming-Brown micropipette puller (P-97; Sutter Instrument, Novato, CA) and I approached the tip of the pipette close to the cells by MP-225 micromanipulator (Sutter Instrument). The resistances of the pipette tips for cell collection were approximately 2–5 MΩ. For collecting the cells in area ventralis telencephali pars dorsalis/supracommissuralis/posterior (Vd/Vs/Vp), nucleus preopticus pars magnocellularis/parvocellularis (POm, POp), and nucleus posterioris periventricularis (NPPv), I sectioned the whole brain frontally by vibratome and the cells were dissociated by Papain solution (Roche Applied Science) and collected likewise under an inverted fluorescence microscope. I

prepared the mixed sample containing 10 cells collected from Vd/Vs/Vp, vPOA, and hypothalamus (mixed 5 cells each from male and female) and performed the following lysis, reverse transcription (RT), and purification steps using SuperScript III (Thermo Fisher Scientific, Waltham, MA) and Nucleospin Gel and PCR clean-up kit (Macherey-Nagel, Berlin, Germany). cDNA libraries were obtained by these procedures mainly based on the standard protocol provided by Life Technologies, and then applied for the next generation sequencer Ion PGM (Life Technologies, Thermo Fisher Scientific), following the standard protocol of Ion PGM system. I selected the candidate genes judging from reads per kilobase of exon per million mapped sequence reads (RPKM) for expression value in the obtained data.

Histological analysis for identification of the candidate genes co-expressed in *gpr54-1* expressing cells

To examine whether *gpr54-1* expressing cells actually co-express the genes detected by deep sequencing, I also performed dual labeling of EGFP IHC and ISH for several candidate genes by using the *gpr54-1*:*EGFP* transgenic medaka. For this analysis, I prepared DIG-labeled sense and anti-sense cRNA probes for each candidate gene (Table 2-1). As for *npb* ISH probes, I prepared them from the plasmid used in Hiraki et al., 2014: a generous gift from Dr. Okubo (the University of Tokyo). I used the same *erα* and *gpr54-1* probes as previously reported (36,46). In this analysis, EGFP immunoreactivities were visualized with Alexa Fluor 488-conjugated streptavidin (1:500;

Invitrogen) after signal-amplification with ABC Elite kit (Vector Laboratories).

On-cell patch recording from vPOA *gpr54-1* expressing neurons

For electrophysiological analyses, I used the *gpr54-1*:*EGFP* transgenic fish. The brains were dissected out and the whole brain preparations were placed onto the bottom of a handmade chamber filled with artificial cerebrospinal fluid (ACSF) consisting of (in mM) 134 NaCl, 2.9 KCl, 1.2 $MgCl_2$, 2.1 $CaCl_2$, 10 HEPES, and 15 glucose (pH 7.4, adjusted with NaOH). Next, I approached a patch pipette to EGFP-positive *gpr54-1* expressing cells in vPOA, using micromanipulator in the same way I performed in sampling for deep sequencing, except that the pipette resistances were approximately 15-25 MΩ. When the tip of the pipette was touched to the surface of the cell, I gave negative pressure using a syringe until the resistance was around 80-150 MΩ. Targeted on-cell patch recordings were performed with a CEZ-2400 patch-clamp amplifier (Nihon Kohden, Tokyo, Japan) or Axopatch 200B, digitized (10 kHz), and stored on a computer using Digidata 1322A and pCLAMP 9.2 or 10.6 software (Molecular Devices, Sunnyvale, CA). During recording, ACSF solution was kept flowing by perfusion system consisting of Dynamax model RP-1 peristaltic pump (Mettler-Toledo Rainin, Oakland, CA), plastic inlet and outlet pipes connected with silicone tube. Various spontaneous action current patterns were recorded while ACSF flowing with or without 1 μM Kiss1-10 in voltage-clamp mode. ACSF with Kiss1 was prepared by adding 1mM Kiss1-10 solution (dissolved in H_2O) and

as a control, ASCF without Kiss1-10 was prepared by adding the same volume of H_2O (vehicle) before application.

Data analysis

All values are shown as mean ± SEM. For electrophysiological analysis, I used clampfit in pCLAMP 9.2 or 10.6 software (Molecular Devices) and compensated waving baselines to detect clear action currents from the raw data. The periods I counted the number of action currents for stastical analysis of records from vPOA Gpr54-1/Npb neurons are as follows: Before; between 6 minutes (min) and 1 min before the timing when ACSF containing 1 μM Kiss1-10 peptide reached the recording chamber. During; between 6 min and 1 min before the timing when ACSF without 1 μM Kiss1-10 peptide reached the recording chamber. Washout; between 5 min and 10 min after the timing when ACSF without 1 μM Kiss1-10 peptide reached the recording chamber. The mean firing frequencies (spikes/seconds) were calculated by dividing the total number of action currents detected during these period by the recording time in each period (300 seconds). Then I compared the values among these periods. For spontaneous activities, I calculated likewise the total number of action currents detected between 3 and 8 min after recording was started or 1 min before applying ACSF containing either 1 μM Kiss1-10 or vehicle (300 seconds). Statistical analyses were performed with Kyplot 5.0 (Kyence, Tokyo, Japan). All microphotographs were processed by Image J software (National Institutes of Health, Bethesda, MD). Photomerge function of

Photoshop (Adobe, San Jose, CA) was used for image stitching.

Results

Establishment of a *gpr54-1*:*EGFP* transgenic medaka line

I successfully screened medaka having *gpr54-1*:*EGFP* transgene in the genome (Figure 2-1A), judging from the presence of EGFP fluorescence in the hearts of the embryos. I observed EGFP fluorescence in the adult brains of F_2 individuals. The EGFP-positive cells were widely located in the telencephalon and the hypothalamus regions, which is consistent with our previous results of *in situ* hybridization (ISH) (36). I also found clear EGFP-positive fibers from POA to the pituitary. In order to confirm the specificity of EGFP expression in *gpr54-1* expressing neurons, I performed dual labeling of EGFP immunohistochemistry (IHC) and *gpr54-1* mRNA ISH. By this analysis, I clearly showed that almost all of these two signals were co-localized in most of the brain regions. Especially, all EGFP expressing cells in ventralis telencephali pars dorsalis/supracommissuralis/posterior (Vd/Vs/Vp), ventrolateral preoptic area (vPOA), and preopticus pars magnocellularis (POm), were *gpr54-1* mRNA positive, which ensures that there was no ectopic EGFP expression in these areas (Figure 2-1B-D). On the other hand, neurons in habenula, which had been shown to express *gpr54-1* (36), lacked EGFP labeling. It is therefore suggested that 3 kb 5' flanking region of *gpr54-1* gene contains essential enhancers, which induce its expression for Vd/Vs/Vp, vPOA, and POm cells, but not for habenula cells. Thus, I succeeded

in establishing a *gpr54-1*:*EGFP* transgenic medaka line for the analysis of *gpr54-1* expressing cells that are localized in the telencephalic and hypothalamic nuclei.

vPOA population of *gpr54-1* expressing cells project their axons to the pituitary

To examine the properties of Kiss1 target neurons, I used the *gpr54-1*:*EGFP* transgenic medaka for multidisciplinary analyses including histology, deep sequencing, and electrophysiology. First, to examine neuronal projection of each population of *gpr54-1* expressing cells in the brain, I performed single EGFP IHC by chromogenic labeling with DAB and observed cell bodies and fibers using sagittal (Figure 2-2A, B) and frontal sections (Figure 2-2C, D). I also performed whole-mount IHC by fluorescent labeling to examine their neuronal projection in the whole brain with pituitary (Figure 2-2E). It should be noted that EGFP passively diffuses in the cytoplasm, and thus EGFP IHC visualizes the whole structure of the neurons, including axon and dendrites. The IHC revealed the whole projection sites and localization of *gpr54-1* expressing cells more clearly than the EGFP fluorescence alone. Notably, it was shown that vPOA population heavily projects to the pituitary, especially in the pars distalis and pars intermedia (Figure 2-2D, E, Figure 2-3G). On the other hand, Vd/Vs/Vp and POm populations spread their fibers in the rostro-caudal direction (Figure 2-2A, Figure 2-3), and EGFP-immunoreactive (EGFP-ir) fibers were also detected from the hypothalamus to the medulla oblongata (Figure 2-2A, Figure 2-3) and even in the spinal cord (data not shown). These data indicate that the vPOA population plays

some roles in the regulation of endocrine functions in the pituitary, and the Vd/Vs/Vp and POm populations may play some roles in the neuromodulation of various functions including behavior.

gpr54-1 expressing neurons co-express neuropeptides such as Npb

To identify co-expressing genes coding neurotransmitters and receptors in *gpr54-1* expressing cells, I performed deep sequencing followed by histological analysis using the *gpr54-1*:*EGFP* transgenic medaka. Deep sequencing of *gpr54-1* expressing cells (Total reads: 428,309, Mean read length: 94 bp, Aligned base rate: 84%) showed about 4000 genes that are possibly co-expressed with *gpr54-1* from RPKM (reads per kilobase of exon per million mapped sequence reads) values. Among them, I detected several coding genes for neuropeptides, such as neuropeptide Y b (NPYb), cholecystokinin (Cck), and neuropeptide B (Npb) (RPKM value; *gpr54-1*: 56.108, *npyb*: 103230.74, *cck*: 1849.84, *npb*: 96.658). *npyb* is the paralog of *npy* (*npya*) reported recently (74), and Npb is one of the novel neuropeptides identified recently (47,75,76). In mammals, NPB has been suggested to be involved in various physiological functions including feeding, social behavior, and endocrine regulation (47,50,77,78). In addition, some genes coding a receptor besides *gpr54-1*, such as estrogen receptor α (ERα) were also detected (RPKM value; *erα*: 8.092). In contrast to the detected candidate genes that are possibly regulated by Kiss1 signals, none of the GnRH related genes (*gnrh1*/*2*/*3*, and *gnrhr1*/*2*/*3*/*4*) was detected by deep sequencing. It is consistent with our previous morphological analysis that GnRH1 neurons lack *gpr54-1*

mRNA expression (36).

Next, I examined the co-expression of these candidate genes and *gpr54-1* by dual labeling of EGFP IHC and ISH. In Vd/Vs/Vp, *npyb*, *cck*, *npb*, and *erα* mRNA signals were clearly co-localized with EGFP-ir signals (Figure 2-4), while in vPOA, only *npb* mRNA signals showed clear co-localization with EGFP-ir signals (Figure 2-5A-F). It should be noted that distinct *npyb* and *cck* mRNA expressions were not detected in vPOA (data not shown). A previous study in medaka demonstrated that *npb* mRNA shows a female-biased expression in several nuclei in telencephalon and hypothalamus including Vd/Vs/Vp but not vPOA (79). Consistent with this, I observed the co-localization of *npb* and Gpr54-1 in both females and males in vPOA (Figure 2-5A-F). As for *erα*, I observed clear co-expression with Gpr54-1 in Vd/Vs/Vp (Figure 2-4J-L), while the expression was nearby EGFP-ir cells in vPOA (Figure 2-5G-I). Here, I successfully identified neurotransmitters for each population (Vd/Vs/Vp: Npyb, Cck and Npb (Figure 2-4), vPOA: Npb (Figure 2-5)). As the EGFP-ir neurons express *gpr-54-1* (Figure 2-1B), it follows that their neural activities should be regulated by Kiss1 neurons. It should be noted that both Vd/Vs/Vp and vPOA populations express Npb as their neurotransmitter and each population projects to different brain regions (Figure 2-2, 2-3). Therefore, these two populations may regulate different kisspeptin functions via Npb.

Neuronal activities of vPOA *gpr54-1* expressing cells are facilitated by Kiss1

To examine the spontaneous activities of *gpr54-1* expressing cells in vPOA that were proven above to be hypophysiotropic Npb neurons (Gpr54-1/Npb neurons), and the effect of Kiss1 peptide (Kiss1-10) on them, I performed on-cell patch clamp analysis of these neurons. vPOA Gpr54-1/Npb neurons showed various spontaneous activities with their firing frequency of 1.01±0.093 Hz (n=77 from 36 fish; Figure 2-6A, B), regardless of sex (Figure 2-6A) and time of day of recording (Figure 2-6B). Furthermore, Kiss1-10 applications by perfusion facilitated their firing activities in most cases (Figure 2-6C-E). Besides, it occasionally induced burst firing with their instantaneous frequencies more than 10 Hz (Figure 2-6C, D). These bursting patterns often persisted even during washout (normal ACSF perfusion) for more than 30 minutes. Comparisons of the firing frequencies during Kiss1-10 perfusion with those of control vehicle showed significant difference (vehicle, n=18 from 16 fish; Kiss1-10, n=22 from 21 fish; $p<0.01$, Mann–Whitney *U* test) (Figure 2-6E). On the other hand, the normalized firing frequencies after washout did not show significant difference compared to those of control vehicle group (vehicle, n=11 from 10 fish; Kiss1-10, n=17 from 16 fish; Mann–Whitney *U* test) (Figure 2-6E), although the potentiated firing activities were often persistent even during washout. These data suggest that Kiss1 application significantly facilitated firing activities of vPOA Gpr54-1/Npb neurons.

Pituitary-projecting axons of vPOA Gpr54-1/Npb neurons are intertwined with those of IT/VT neurons

To search for targets of vPOA Gpr54-1/Npb neurons in the pituitary, I next performed dual labeling by EGFP IHC and IHC or ISH of several pituitary hormones using the *gpr54-1*:*EGFP* transgenic medaka. I hypothesized that, among several hormone-expressing cells, isotocin (IT) and vasotocin (VT) neurons can be potent candidates of the targets of Kiss1 signals partly because both IT and VT neurons in POA are hypophysiotropic and suggested to be directly regulated by Kiss2 in medaka (36,80). Interestingly, I found a notable characteristic that axons of vPOA Gpr54-1/Npb neurons and IT/VT neurons are intertwined in the pars distalis and pars intermedia, but not in the pars nervosa of the pituitary (Figure 2-2D, E, Figure 2-7). Particularly, axons of Gpr54-1/Npb neurons appeared to surround the varicosities of IT/VT neurons there, suggesting axon-axonal association for regulating IT/VT release (Figure 2-7C, D). This characteristic morphology is unique and, to my knowledge, has never been reported so far.

Discussion

Novel functions of kisspeptin neuronal system revealed by visualization of its receptor-expressing cells

As described above, accumulating body of evidence from studies on possible kisspeptin functions using teleosts has revealed that kisspeptin neuronal system in teleosts is not involved in

the HPG axis regulation. On the other hand, comparative studies on kisspeptin neuronal system have demonstrated that kisspeptin-related genes and estrogen sensitivity of hypothalamic kisspeptin neurons have been widely conserved except birds. Thus, it has been generally accepted that kisspeptin neurons are highly sensitive to estrogen and drastically change their kisspeptin expression in accordance with the breeding states, which may be conserved in vertebrates (18-22,30,44-46,69). Therefore, it is important to search for the estrogen-dependent unknown functions of kisspeptin neuronal system other than the HPG axis regulation. In the present study, I generated the first transgenic line whose kisspeptin receptor-expressing cells are labeled by EGFP (Figure 2-1). Although projection sites of minor populations of Gpr54-1 neurons in the caudal brain regions that were observed in this transgenic medaka (Figure 2-3H, I) might be an ectopic EGFP expression considering the previous *gpr54-1* ISH study (36), the specificity of EGFP-positive fibers originating from Vd/Vs/Vp, vPOA, and POm was confirmed by detailed microscopic observations (Figure 2-2, Figure 2-3A-G). Thus, I can tell for sure that all the fibers shown in Figure 2-2B-E originate from these validated nuclei. As shown here, *gpr54-1* expressing cells in Vd/Vs/Vp, vPOA, and POm were specifically visualized with EGFP in the live brain, which was highly useful for the multidisciplinary analyses in the present study: histology of axonal projections, deep sequencing and subsequent identification of neurotransmitters, and electrophysiology of spontaneous and evoked activities by Kiss1-10. It should be noted that, unlike the previous studies, the present approach successfully visualized the whole morphology

of Gpr54-1 neurons by EGFP, which was applied to comprehensive analyses. Surprisingly, EGFP IHC analysis of the axonal projection demonstrated a dense pituitary-projecting axon bundle that originates from the vPOA population (Figure 2-2B-E). By combination of deep sequencing and histological analysis, I identified Npyb, Cck, and Npb as their neurotransmitters (Figure 2-4A-I). A previous study using European sea bass has also suggested that *gpr54-2* mRNA is expressed in the neurons expressing Npya, somatostatin, dopamine, and nitric oxide (NO) (35). However, the regulation of these neurons is probably independent of reproductive states considering that Kiss2 neurons in European sea bass lack ERα and they are estrogen-insensitive (81), and that Gpr54-2 is activated by estrogen-insensitive Kiss2 neurons in medaka (45,46). Thus, I demonstrated that Gpr54-1 neurons express non-GnRH neuropeptides such as Npb, which is considered to be regulated by Kiss1 peptide. To examine whether Kiss1 facilitates their firing activities and possible peptide release, I next performed electrophysiological analysis of vPOA Gpr54-1/Npb neurons. On-cell patch clamp recording suggested that Kiss1-10 facilitates their neuronal activities and often causes high frequency bursting. It has been reported that such high frequency bursting causes peptide release (82,83). Therefore, it is suggested that Kiss1-induced bursting stimulates Npb release from these vPOA Gpr54-1/Npb neurons.

In conclusion, my multidisciplinary analyses strongly suggest that vPOA Gpr54-1/Npb neurons release Npb in response to Kiss1 signals during the breeding condition and regulate some endocrine functions in the pituitary.

Identification of hypophysiotropic neuropeptide B neuronal system and its possible regulation by kisspeptin neuronal system

Neuropeptide B, NPB and NPW (product of a *npb* paralog, *npw*) are novel neuropeptides recently identified as endogenous ligands for Gpr7 and Gpr8, and are widely conserved in vertebrates (47,50,75,76,78). Many previous studies reported in mammals that NPB and NPW are involved in various physiological functions such as feeding, endocrine, and stress response (47,50,76-78), although the regulatory mechanisms remain largely unclear. In the present study, I demonstrated that Npb neurons in both Vd/Vs/Vp and vPOA express *gpr54-1*. Besides, previous studies suggested that Vd/Vs/Vp region is involved in reproductive behaviors (84-87), and that *npb* mRNA expression in Vd/Vs/Vp is female-specific, which implies sexual dimorphism in Vd/Vs/Vp Npb neurons and their involvement in the control of female-specific reproductive behaviors (79). In addition, because the Gpr54-1 expressing neurons in Vd/Vs/Vp also co-expressed *erα* mRNA (Figure 2-4J-L) they can be regulated by both kisspeptin and gonadal estrogen. This is consistent with the previous study that showed expression of *erα* mRNA in these female-specific Npb neurons in Vd/Vs/Vp (79) (Figure 2-4G-I, 6A-F). I also suggested histologically that Vd/Vs/Vp population co-express *npyb* or *cck* in addition to *npb* (Figure 2-4A-I). Interestingly, all these neuropeptides are suggested to be involved in feeding in both teleosts and mammals in several previous studies (50,88-90). Thus, I suggested for the first time that kisspeptin neuronal system regulates various functions including reproductive behavior and/or

feeding behavior via various neural systems from these distinct populations.

Another Gpr54-1/Npb population localized in vPOA were proven to be hypophysiotropic. To date, only few hypophysiotropic peptidergic neurons have been identified, and they are suggested to be involved in crucial endocrine functions such as GnRH, corticotropin-releasing hormone (CRH), and growth hormone releasing hormone (GHRH) neurons (52). Thus, vPOA Gpr54-1/Npb population may regulate some important endocrine functions in the pituitary. A previous i.p. administration study suggested in Nile tilapia that Npb affects prolactin and growth hormone expressions in the pituitary (77). In addition to this, I histologically suggested that IT and VT neurons in POm that are hypophysiotropic and release IT or VT in the pituitary (80) have axon-axon interactions with vPOA Gpr54-1/Npb neurons in the pars distalis (Figure 2-7). IT or VT neuronal projection to the pars distalis are suggested to regulate hormone secretion from the pituitary endocrine cells, while that to the pars nervosa directly regulates peripheral tissues via general circulation (52). Taken together, these hypophysiotropic Npb neurons can regulate pituitary hormonal release by changing IT and/or VT release in addition to the possible direct regulation on the pituitary endocrine cells.

In fact, IT and VT have been suggested to be involved in many endocrine and behavioral functions such as stress response (91,92) or behaviors corresponding to social information (51,93-95) in various vertebrates. Previous studies showed that IT and VT neurons can be also regulated by Kiss2 via Gpr54-2 (36) and that Gpr8 is a G_i-coupled GPCR (50,75,76,78). Taken together,

these lines of evidence suggest that the hypophysiotropic Kiss1-Npb neuronal pathway possibly inhibits IT and VT release at axon terminals in the pituitary. Considering the high estrogen-sensitivity of Kiss1 neurons (44,45), kisspeptin neuronal system may play various physiological roles via Npb, IT and VT and contribute to breeding state-specific functions for successful reproduction.

Search for evolutionary common functions of kisspeptin neurons

In summary, my present strategy of visualizing receptor-expressing neurons and using them for multidisciplinary analyses, succeeded in demonstrating that kisspeptin neuronal system regulates various peptidergic systems other than HPG axis. Particularly, I suggest a series of physiological pathways from the gonads to the pituitary regulated by kisspeptin neuronal system (Figure 2-8). Here, gonadal estrogen facilitates NVT Kiss1 neuron activities, and in turn, Kiss1 signals inhibit IT and VT neurons, which is mediated by vPOA Gpr54-1/Npb neurons. Thus, I suggest that gonadal estrogen regulates pituitary hormone release through this neural pathway.

The present and previous studies showed consistent results that kisspeptin is not involved in the HPG axis regulation among evolutionally distant teleost species such as goldfish, medaka, zebrafish, European sea bass, and African cichlid. Considering such consistent results and the high estrogen-sensitivity of kisspeptin neurons probably conserved among vertebrates (18-22,30,44-46,69), it is speculated that, before the emergence of mammals, kisspeptin used to

contribute to non-reproductive functions in response to serum sex steroids. In mammals, GnRH neurons happened to start expressing Gpr54, which led to the kisspeptin-dependent HPG axis regulation system.

All neuropeptides mentioned in the present study are widely conserved in vertebrates (note that IT and VT are peptides coded by orthologs of mammalian oxytocin (OT) and vasopressin (VP) respectively). Interestingly, recent studies in mammals also demonstrated interactions between kisspeptin and NPB/NPW, NPY, VP and OT (96-100). This suggests that kisspeptin regulation of these neuropeptides may be evolutionarily conserved regulatory pathways of kisspeptin neural system common to vertebrates.

The present study gives various neuroendocrinological insights into how the regulatory mechanism of reproduction has evolved in vertebrates.

Conclusions

In this chapter, I discovered that various non-GnRH neuropeptidergic systems are involved in the regulation by the Kiss1-Gpr54-1 neuronal system. Among them, vPOA Gpr54-1/Npb neurons, which can be regulated by estrogen-dependent Kiss1 signaling, were suggested to regulate IT/VT neurons in pars distalis and pars intermedia of the pituitary. Therefore, these data suggest the neuronal pathways between Kiss1-Gpr54-1 neuronal system and IT/VT neurons… (5 年以内に雑誌等で刊行予定のため、非公開。)

Figure legends

Figure 2-1.

Establishment of a *gpr54-1*:*EGFP* transgenic medaka and their specific EGFP expression in *gpr54-1* expressing cells. A, The construct used to generate the transgenic medaka. EGFP-coding sequence and polyadenylation signal (SV40 polyA) were fused to the approximate 6 kb DNA fragment containing the 5' -flanking region of *gpr54-1* gene (3 kb). The construct contains pcMLC2 enhancer to express EGFP in the heart for efficient screening in the embryonic stages. B-D, Dual labeling of EGFP Immunohistochemistry (IHC) (left column) and *gpr54-1* mRNA *in situ* hybridization (ISH) (middle column). Overlay of both signals (right column) shows that all EGFP-ir (immunoreactive) cell bodies in the Vd/Vs/Vp (B), POA (C), and POm (D) were *gpr54-1* mRNA positive. Scale bars, 20 μm (B-D).

Figure 2-2.

Visualization of *gpr54-1* expressing cells by EGFP IHC in the *gpr54-1*:*EGFP* transgenic medaka shows hypophysiotropic neuronal projection from vPOA. A-D, Light microphotographs showing the representative serial sagittal (A, B) and frontal (C, D) sections of EGFP-ir (*gpr54-1* expressing) cells in the brain and pituitary of the transgenic medaka. EGFP signals were visualized with DAB. A, EGFP-ir cell bodies were mainly localized in Vd/Vs/Vp, POA, and some regions in hypothalamus. B, A dense axon bundle of vPOA *gpr54-1* expressing cells projects from vPOA (white arrowheads in A and B) to the pituitary (black arrowheads in A and B). C, Cell bodies of *gpr54-1* expressing cells in vPOA. D, Axon terminals of vPOA *gpr54-1* expressing cells in the pars distalis of the pituitary. These histological observations suggest that fibers from vPOA population heavily project to the pituitary (B-D), while the other populations project widely in rostral and caudal directions (A). E, Representative fluorescent microphotograph showing EGFP-ir fibers project to the pituitary. The dotted line and the arrow indicate the outline of the pituitary

and the EGFP-ir axon bundle, respectively. A; Anterior, P; Posterior, D; Dorsal, V; Ventral. Scale bars, 200 μm (A), 100 μm (B, E) or 50 μm (C, D). C-D, counterstained by Nissl.

Figure 2-3.

Schematic illustrations showing the projection of *gpr54-1* expressing cells in the brain depicted from sequential frontal sections visualized by EGFP IHC. Cell bodies of EGFP-ir (Co-expression with *gpr54-1* mRNA is confirmed) cells are localized in Vd/Vs/Vp (A-E), vPOA (C-E), and POm (E, F). Detailed observation of serial sections strongly suggested that the projection of *gpr54-1* expressing cells in the vPOA was almost confined to POA (C-E) and the pituitary (G), while that of the other populations widely spread in ventral telencephalon, POA, and hypothalamus. Projection sites of minor populations of Gpr54-1 neurons in the caudal brain regions were observed (*gpr54-1* mRNA has not been detected by ISH) (H, I). EGFP-ir fibers were also observed in the medulla oblongata (I), although it is difficult to distinguish where they were derived from. The nomenclature of the medaka brain nuclei: ca/ch/cp/ct, commissura anterior/horizontalis/posterior/transversa; CE, corpus cerebelli; Dc/Dm/Dl/Dp, area dorsalis telencephali pars centralis/medialis/lateralis/posterior; DM, nucleus dorsomedialis thalami; flm, fasciculus longitudinalis medialis; fr, fasciculus retroflexus; GR, corpus glomerulosum pars rotunda; HB, habenula; lfb, lateral forebrain bundle; mfb, medial forebrain bundle; NAT, nucleus anterior tuberis; NC, nucleus corticalis; NDTL, nucleus diffusus tori lateralis; NIP, the interpeduncular nucleus; NPPv, nucleus posterioris periventricularis; NVT, nucleus ventralis tuberis; PGm, nucleus preglomerulosus pars medialis; POm/POp, nucleus preopticus pars magnocellularis/parvocellularis; pTGN, preglomerular tertiary gustatory nucleus; PTH, nucleus prethalamicus; TO, tectum opticum; TL/TS, torus longitudinalis/semicircularis; Vd/Vs/Vp/Vi/Vv, area ventralis telencephali pars dorsalis/supracommissuralis/posterior/intermedia/ventralis VM, nucleus ventromedialis thalami.

Figure 2-4.

Dual fluorescence labeling of IHC and ISH showing co-expression of the candidate genes and *gpr54-1* expressing cells in Vd/Vs/Vp. Representative microphotographs of frontal sections in Vd/Vs/Vp by dual labeling of EGFP IHC and ISH for co-expressing candidate genes (*npyb*, *cck*, *npb*, *erα*) using the *gpr54-1*:*EGFP* transgenic medaka brain. EGFP-ir *gpr54-1* expressing cells (A, D, G, J; green) co-express the candidate genes, *npyb*, *cck*, *npb*, and *erα* (B, E, H, K, respectively; magenta). Overlay photographs are shown in C, F, I, and L, respectively. Scale bars, 50 μm.

Figure 2-5.

Dual fluorescence labeling of IHC and ISH showed that Npb neurons express *gpr54-1* in vPOA in both sexes, while *gpr54-1* expressing cells do not co-express *era* in vPOA. Representative microphotographs of frontal sections in vPOA by dual labeling of EGFP IHC and ISH for *npb* and *erα* using the *gpr54-1*:*EGFP* transgenic medaka brain. EGFP-ir *gpr54-1* expressing cells in vPOA (A, D; green) co-express *npb* (B, E; magenta) in both female (A-C) and male (D-F). Overlay photographs are shown in C and F respectively. On the other hand, *erα* mRNA signals (H; magenta) do not express in *gpr54-1* expressing cells in vPOA (G; green). Overlay photographs are shown in I. The dotted line in I represents median. Scale bars, 50 μm.

Figure 2-6.

Electrophysiological analysis of vPOA Gpr54-1/Npb neurons by on-cell patch clamp recording shows their various spontaneous activities and facilitation by Kiss1. A, The scatter and box-and-whisker plot showing the spontaneous firing frequencies of vPOA Gpr54-1/Npb neurons. The average firing frequency was 1.01±0.093 (mean ± SEM) and there was no significant difference between males and females (male; n=37, white circles, female; n=38, black circles, Mann–

Whitney *U* test). B, The box-and-whisker plot showing the average spontaneous firing frequencies of vPOA Gpr54-1/Npb neurons recorded at various time-of-day. There was no significant difference among these recording periods (12-3 PM; n=23, 3-6 PM; n=35, 6-10 PM; n=17, Steel-Dwass test). C, Representative traces from one record (1 min each) showing facilitation of the firing activity by Kiss1-10 application. D, The scatter plot of the record shown in C. Instantaneous frequencies of this record drastically increased during 1 μM Kiss1-10 application (The black bar). The colored columns represent the periods used for the analysis shown in E (Gray; Before, Red; 1 μM Kiss1-10, Magenta; Wash out). E, The scatter and box-and-whisker plot showing firing frequencies of vPOA Gpr54-1/Npb neurons normalized with those before 1 μM Kiss1-10 or vehicle application (Dotted line, 100 %) from multiple records. The normalized firing frequencies were significantly increased by 1 μM Kiss1-10 application compared to the vehicle group (vehicle; n=18, 1 μM Kiss1-10; n=21, Mann–Whitney *U* test). There was no significant difference in those in washout (vehicle; n=11, 1 μM Kiss1-10; n=17, Mann–Whitney *U* test).

Figure 2-7.

Dual fluorescence labeling of EGFP and IT or VT by IHC showing that vPOA Gpr54-1/Npb neurons and IT/VT neurons are intertwined in their nerve terminals in the pars distalis and pars intermedia of the pituitary. Representative overlay microphotographs of frontal sections in pituitary using the *gpr54-1*:*EGFP* transgenic medaka brain. EGFP-ir axon fibers of Gpr54-1/Npb neurons (A-D; green) are surrounding those of IT neurons (A, C; magenta) and VT neurons (B, D; magenta) in the pars distalis and pars intermedia (lower or right parts of A and B), but not in the pars nervosa (upper or left parts of A and B) of the pituitary. Dotted squares in A and B indicate magnified areas shown in C and D respectively. Scale bars, 50 μm (A, B), 10 μm (C), and 20 μm (D) respectively.

Figure 2-8.

Schematic illustration of possible neural pathways of kisspeptin neuronal system comprised of multiple peptidergic neural systems. Gonadal estrogen facilitates NVT Kiss1 neuron activities via ERα (estrogen receptor α). Next, NVT Kiss1 neurons stimulate vPOA Gpr54-1/Npb neurons whose axon terminals are intertwined with those of IT and VT neurons in the pars distalis and pars intermedia of the pituitary. I hypothesized that Npb may have some regulatory effects on IT/VT secretion there. On the other hand, there can be other pathways of IT or VT neurons regulated by Kiss2, because both IT and VT neurons express *gpr54-2* (36). Regarding Vd/Vs/Vp *gpr54-1* expressing neurons, they may be involved in some feeding regulation by enhancing release of feeding-related peptides in the brain. Note that expression of Gpr54 in GnRH neuron is only observed in mammals so far and is probably not required for non-mammals, because they lack pulsatile LH release. It is suggested that the common ancestor of mammals may have begun to express Gpr54 on GnRH neurons.

Table 2-1.

The list of primers used for Generation of the *gpr54-1*:*EGFP* line and the ISH probes.

Primer name	Purpose	Sequence
gpr54-1 SE	Construction for *gpr54-1*:*EGFP*	5’- GGGAGGCAGCGGTAGGTATG -3’
gpr54-1 AS	Construction for *gpr54-1*:*EGFP*	5’- GAAGCTCTGTCCATCAGCCTATT AAAAACAGACACTTGTATCTCAC -3’
NPY2-SE(SP6)	*npyb* sense probe for ISH	5’- ATTTAGGTGACACTATAGAAC TGTCCATCGCTGGGATGTC-3’
NPY2-AS(T7)	*npyb* antisense probe for ISH	5’- TCTAATACGACTCACTATAGGGCA CAGGCAGACCCTCATACCTG -3’
mf_SP6-CCK_Fw	*cck* sense probe for ISH	5’- TTATTTAGGTGACACTATAGA TCACACGCTCAACTTCTCTCC -3’
mf_T7-CCK_Rev	*cck* antisense probe for ISH	5’- AATTCTAATACGACTCACTATAGG GAACCTTCTCCATCCCAAATGATTAAG -3’

Table 2-2.

The list of primary antibodies used for IHC.

Antigen	**Name**	**Manufacturer (catalog number)**	**Species**	**Dilution used**
oxytocin/ isotocin	RO-2K	Dr. Seiichiro Kawashima	rabbit polyclonal	1:1000
vasopressin/ vasotocin	RV-1K	Dr. Seiichiro Kawashima	rabbit polyclonal	1:1000
EGFP	anti-EGFP antibody	Nacalai Tesque (GF090R)	rat monoclonal	1:1000
EGFP	GFP Tag Antibody	ThermoFisher (A-11122)	rabbit polyclonal	1:1000

Figure 2-1

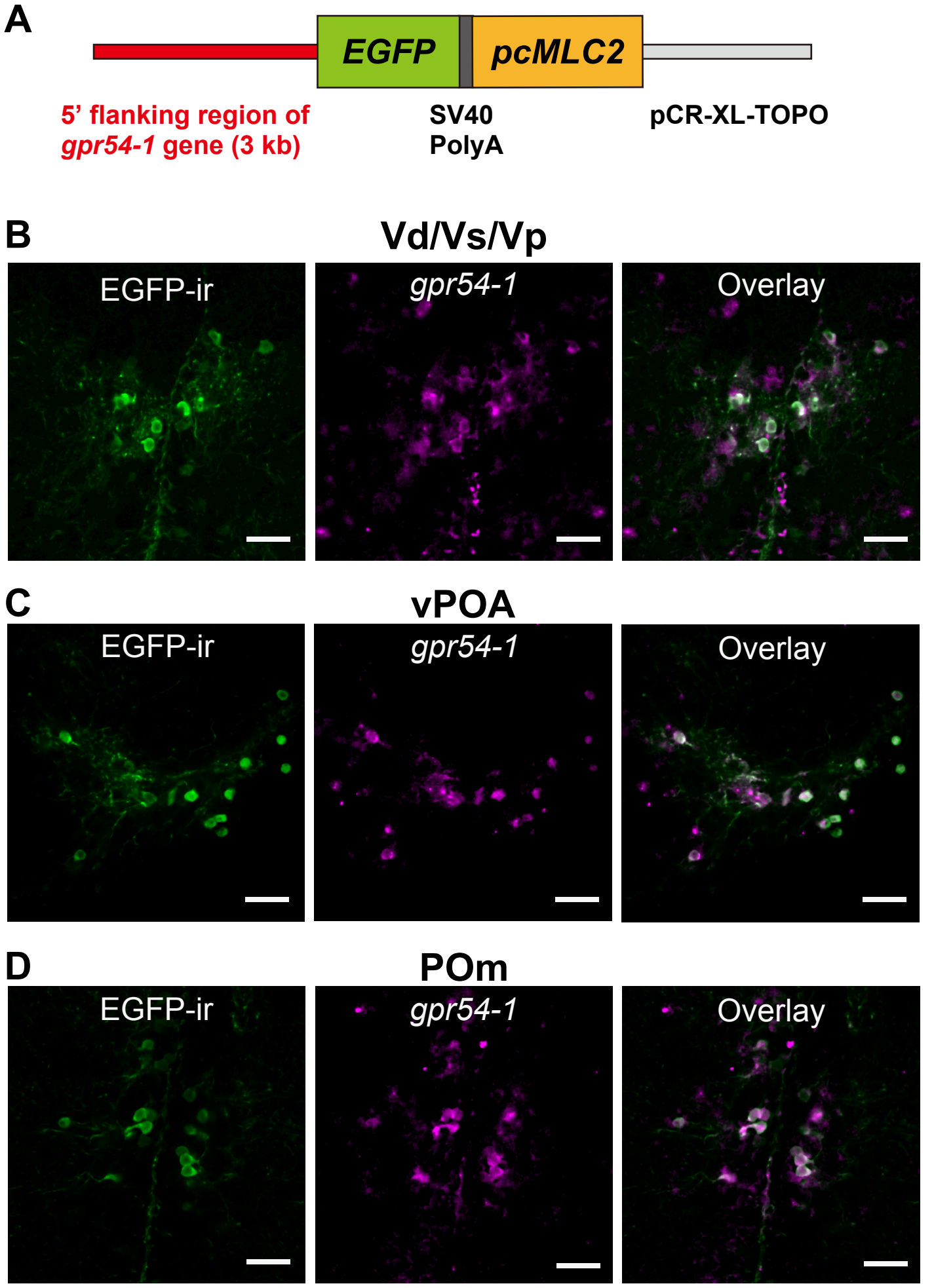

A
EGFP
pcMLC2
5' flanking region of gpr54-1 gene (3 kb)
SV40 PolyA
pCR-XL-TOPO
B
Vd/Vs/Vp
EGFP-ir
gpr54-1
Overlay
C
vPOA
EGFP-ir
gpr54-1
Overlay
D
POm
EGFP-ir
gpr54-1
Overlay

Figure 2-2

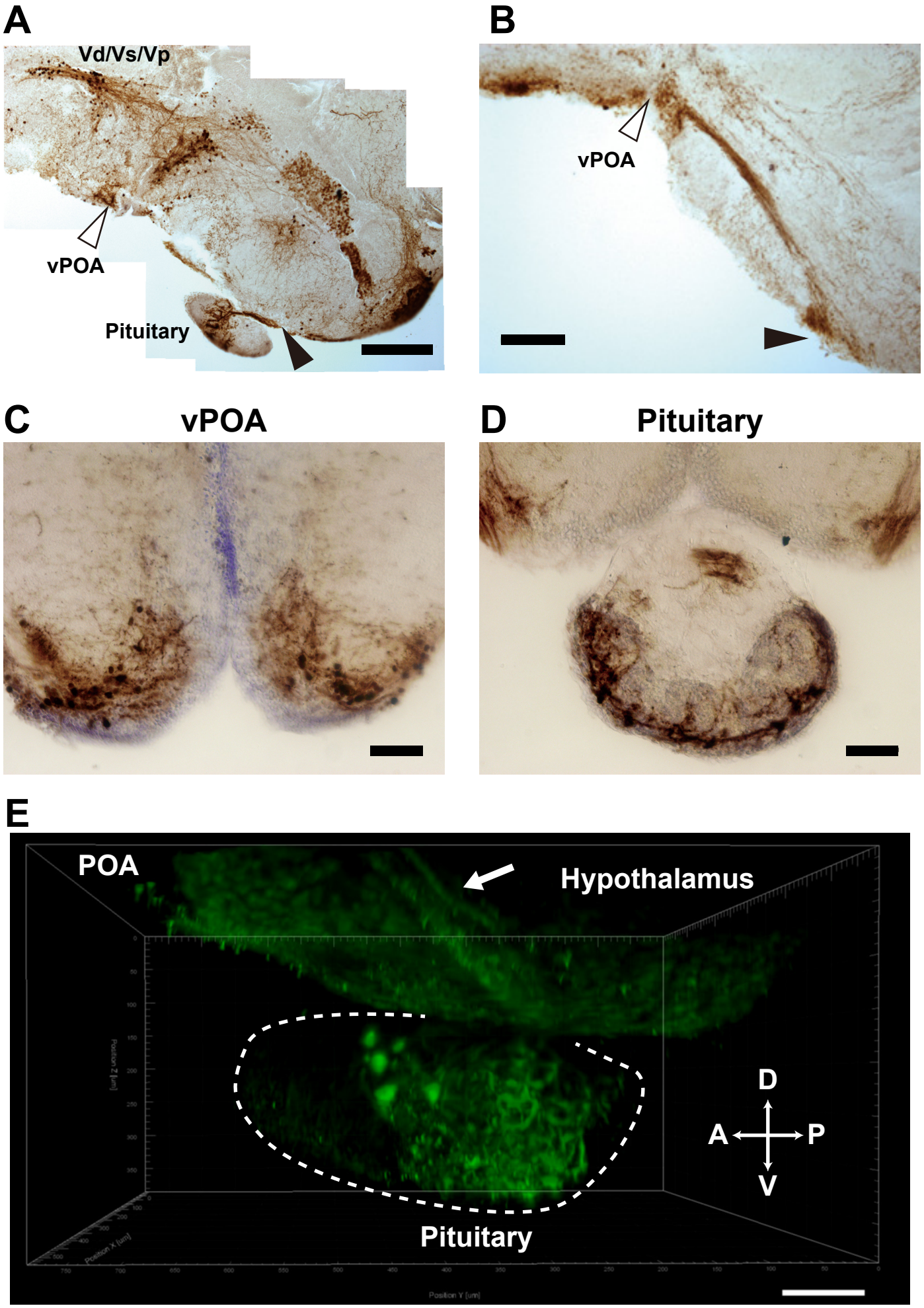

A
Vd/Vs/Vp
vPOA
Pituitary
B
vPOA
C
vPOA
D
Pituitary
E
POA
Hypothalamus
Pituitary
D
A
P
V

Figure 2-3

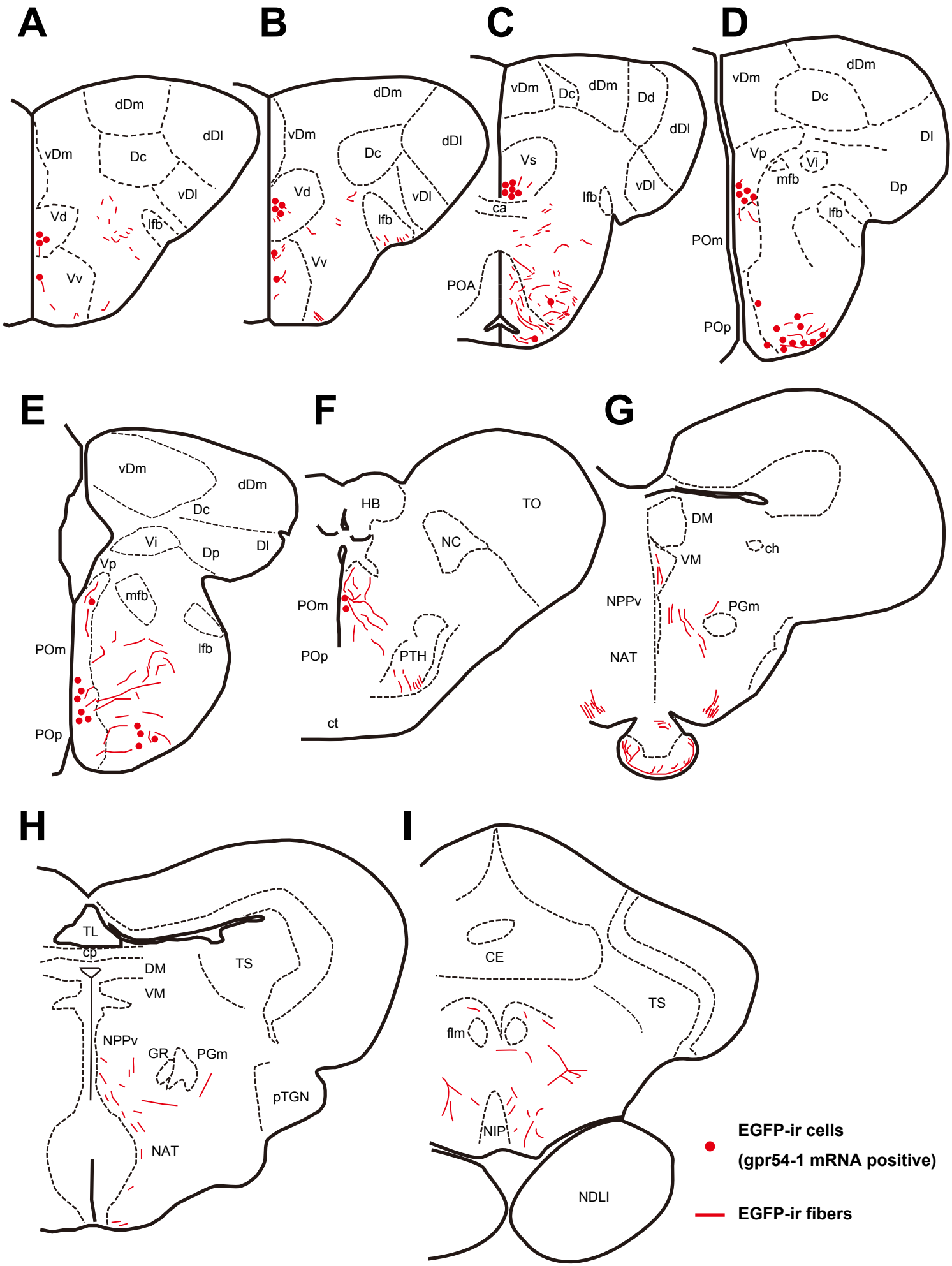

A
dDm
vDm
Dc
dDl
vDl
Vd
lfb
Vv
B
dDm
vDm
Dc
dDl
Vd
vDl
lfb
Vv
C
vDm
Dc
dDm
Dd
dDl
Vs
vDl
lfb
ca
POA
D
vDm
dDm
Dc
Dl
Vp
Vi
mfb
Dp
lfb
POm
POp
E
vDm
dDm
Dc
Vi
Dp
Dl
Vp
mfb
POm
lfb
POp
F
HB
TO
NC
POm
POp
PTH
ct
G
DM
ch
VM
NPPv
PGm
NAT
H
TL
cp
DM
TS
VM
NPPv
GR
PGm
pTGN
NAT
I
CE
TS
flm
NIP
NDLI
EGFP-ir cells
(gpr54-1 mRNA positive)
EGFP-ir fibers

Figure 2-4

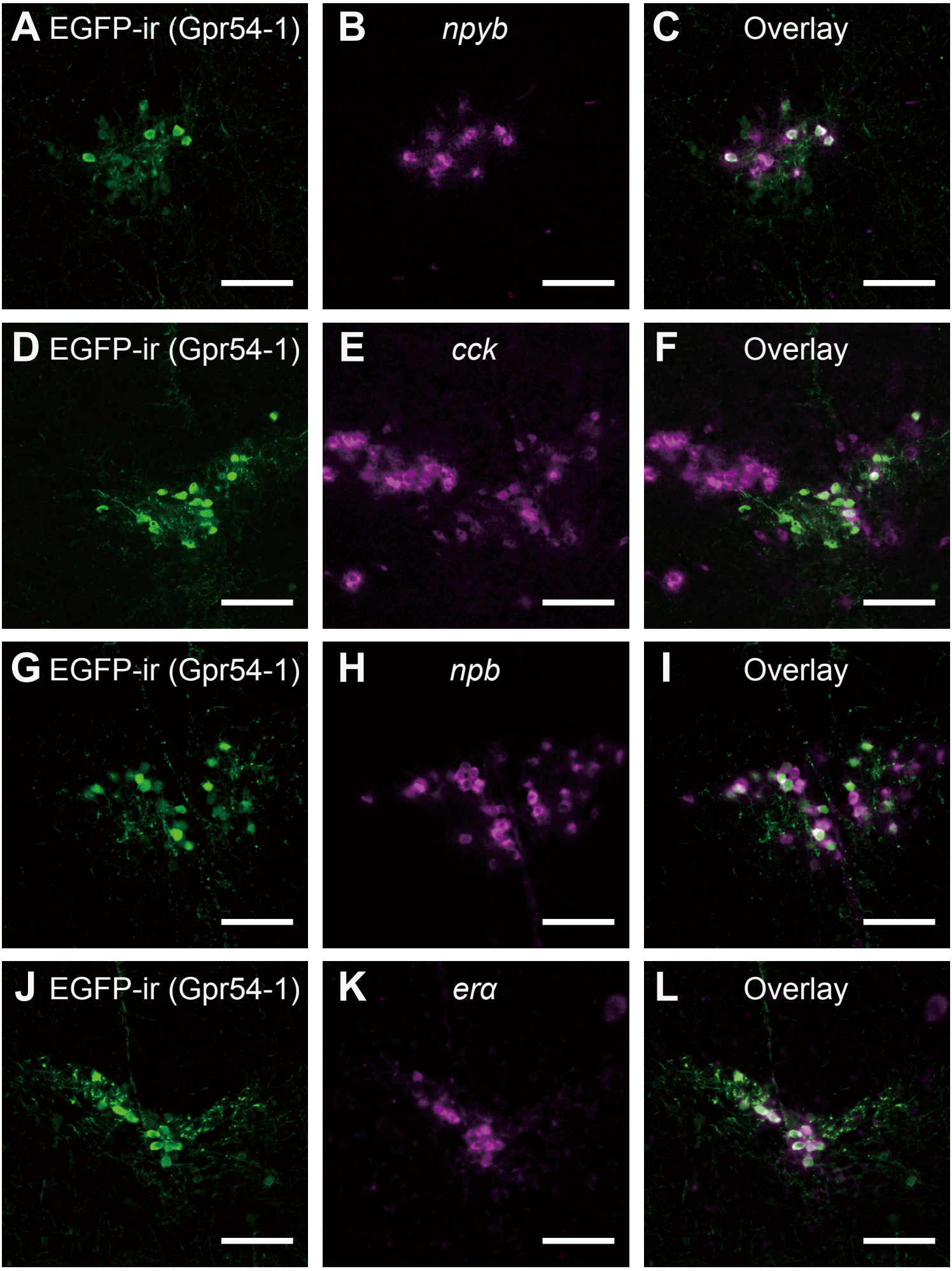

A EGFP-ir (Gpr54-1)
B npyb
C Overlay
D EGFP-ir (Gpr54-1)
E cck
F Overlay
G EGFP-ir (Gpr54-1)
H npb
I Overlay
J EGFP-ir (Gpr54-1)
K erα
L Overlay

Figure 2-5

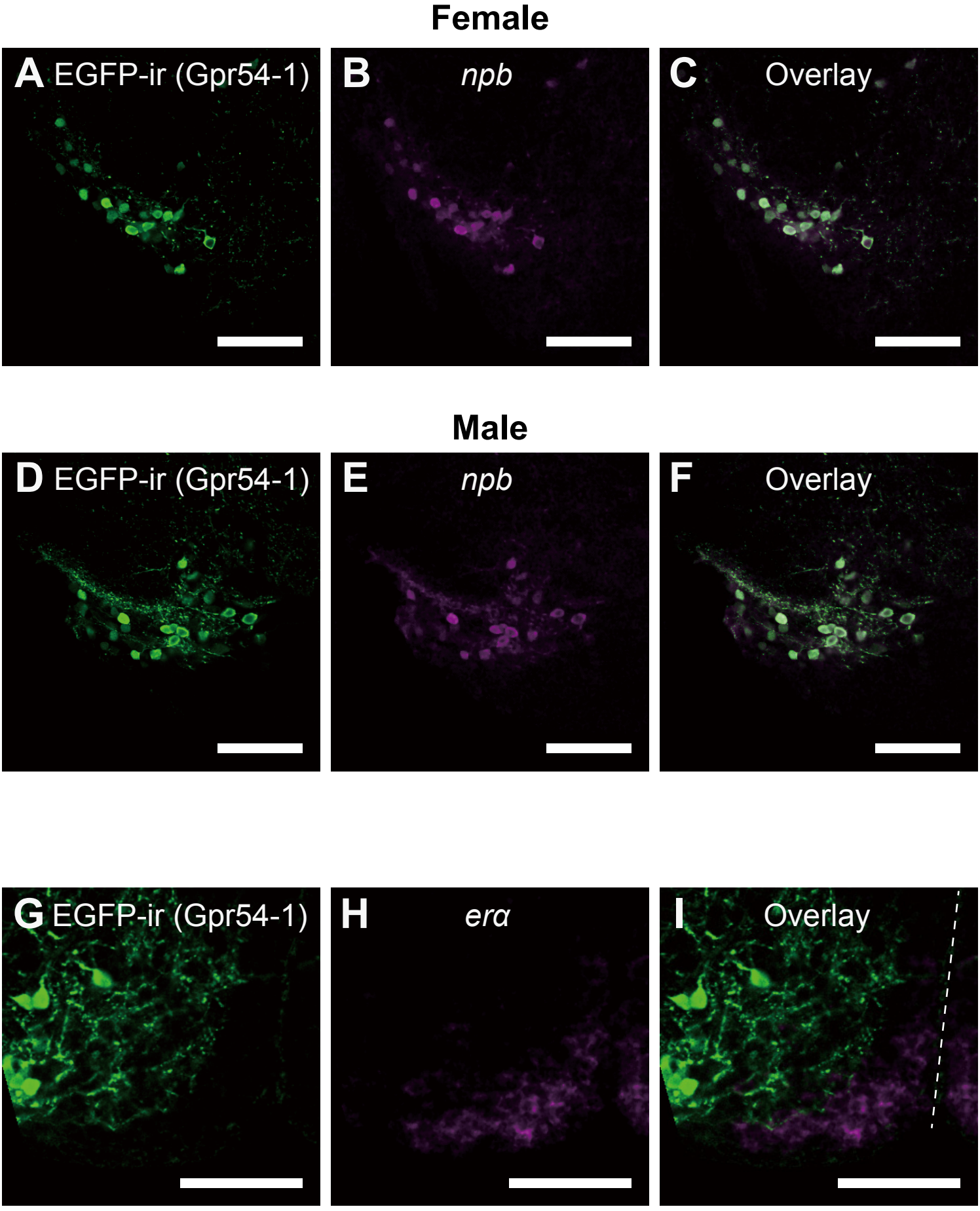

Female
A EGFP-ir (Gpr54-1)
B npb
C Overlay
Male
D EGFP-ir (Gpr54-1)
E npb
F Overlay
G EGFP-ir (Gpr54-1)
H erα
I Overlay

Figure 2-6

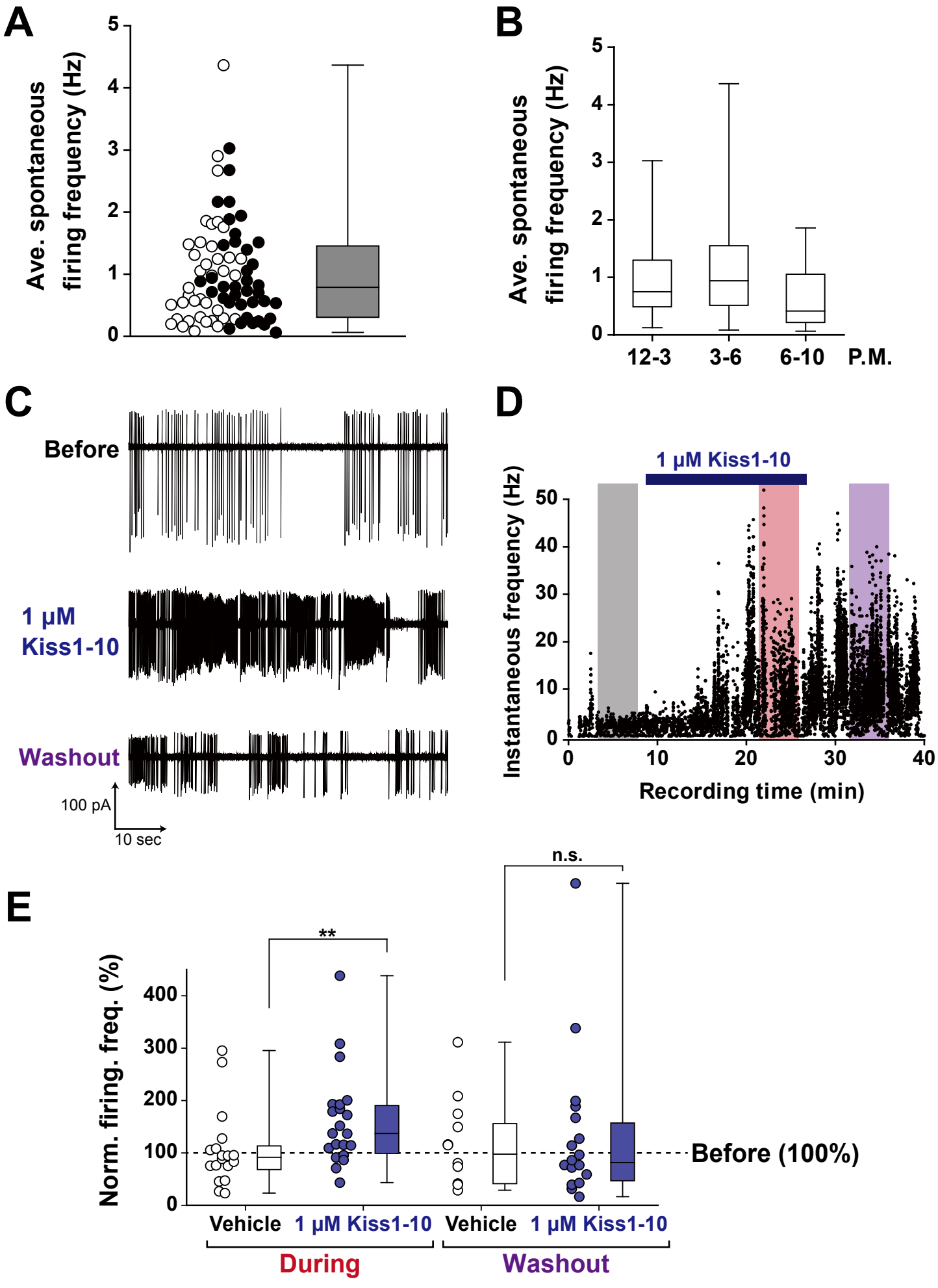

A
Ave. spontaneous firing frequency (Hz)
0
1
2
3
4
5
B
Ave. spontaneous firing frequency (Hz)
0
1
2
3
4
5
12-3
3-6
6-10
P.M.
C
Before
1 μM Kiss1-10
Washout
100 pA
10 sec
D
1 μM Kiss1-10
Instantaneous frequency (Hz)
0
10
20
30
40
50
0
10
20
30
40
Recording time (min)
E
n.s.
**
Norm. firing. freq. (%)
0
100
200
300
400
Before (100%)
Vehicle
1 μM Kiss1-10
Vehicle
1 μM Kiss1-10
During
Washout

Figure 2-7

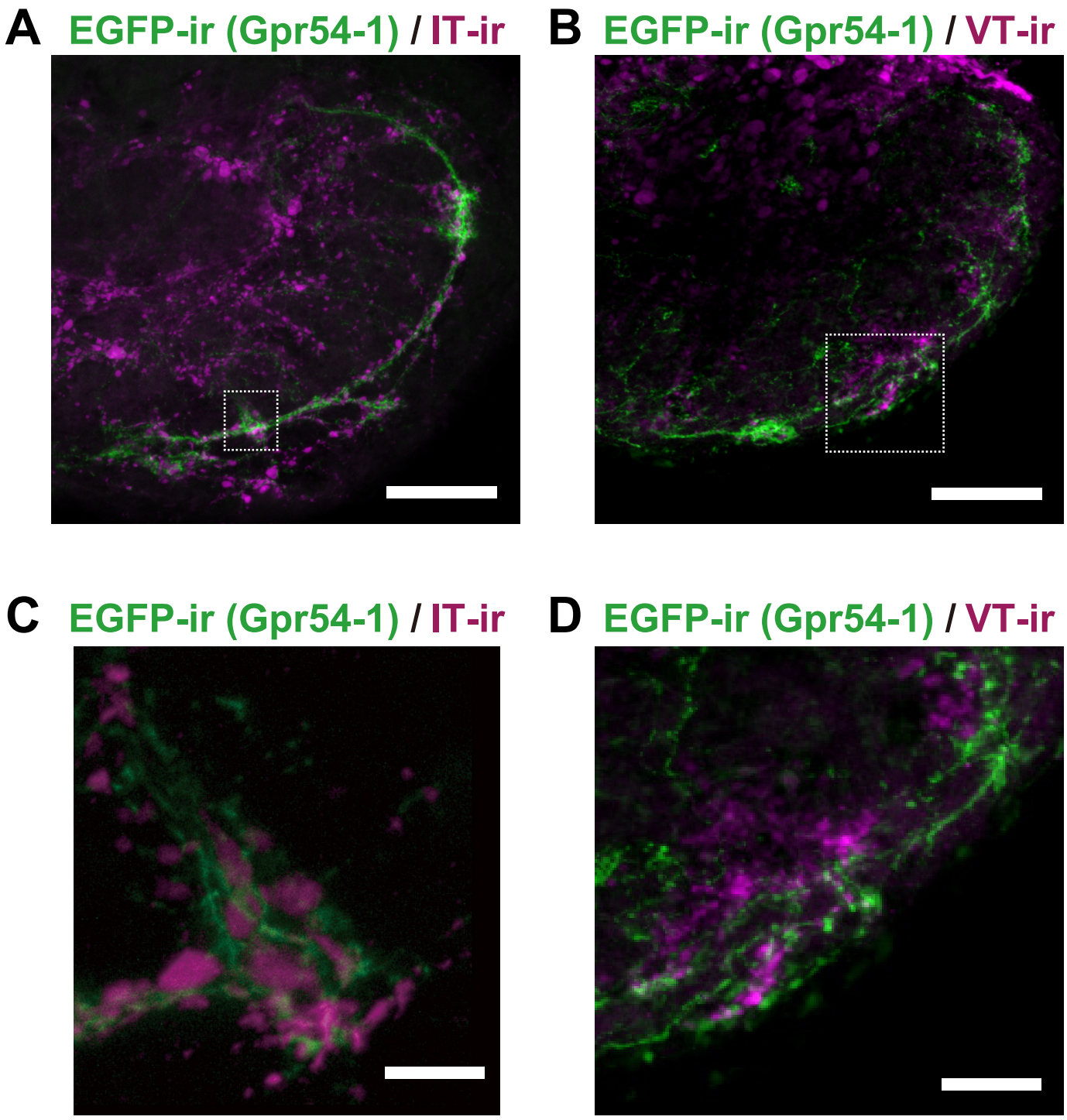
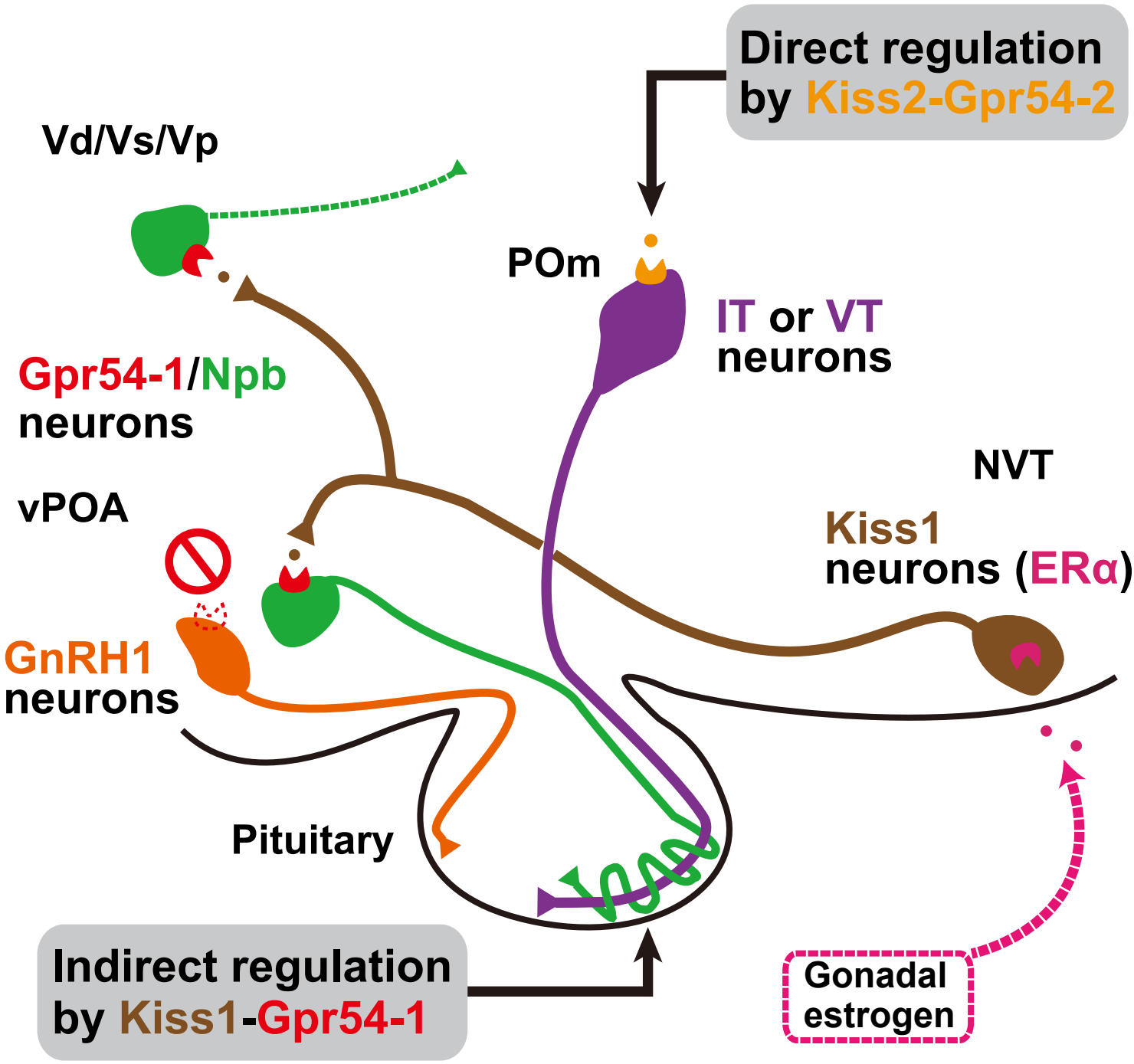

A EGFP-ir (Gpr54-1) / IT-ir
B EGFP-ir (Gpr54-1) / VT-ir
C EGFP-ir (Gpr54-1) / IT-ir
D EGFP-ir (Gpr54-1) / VT-ir

Figure 2-8


Direct regulation
by Kiss2-Gpr54-2
Vd/Vs/Vp
POm
IT or VT
neurons
Gpr54-1/Npb
neurons
NVT
vPOA
Kiss1
neurons (ERα)
GnRH1
neurons
Pituitary
Indirect regulation
by Kiss1-Gpr54-1
Gonadal
estrogen

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

(5 年以内に雑誌等で刊行予定の部分の記述については非公開。)

In the present thesis, I took advantages of medaka as a model animal for the study of functional regulatory mechanisms of reproduction and discovered unidentified novel functions of kisspeptin neuronal system in non-mammalian species, which suggest the kisspeptin regulation of IT/VT neuronal systems via Npb leading to the control of stress response.

In Chapter 1… (5 年以内に雑誌等で刊行予定のため、非公開。)

Since the discovery of kisspeptin in mammals as an essential component of the HPG axis regulation, many studies using non-mammalian species, especially teleosts, have examined possible involvement of kisspeptin in the HPG axis regulation (34-37,39-41,45,81,101-108). However, their results have been controversial and there has been no general consensus until recent studies clearly denied the involvement in the HPG axis regulation in teleosts (34-37,43). In addition, avian species have lived to produce their offspring without kisspeptin genes. Thus, it is speculated that the kisspeptin function as an essential HPG-axis regulator is only limited to mammalian species. On the other hand, kisspeptin-related genes are widely conserved among almost all vertebrate species except birds, suggesting existence of some important functions of kisspeptin that is related to some aspects of reproduction (27,29,31,33). However, due to the technical limitations, such important functions of kisspeptin has not been comprehensively studied and the underlying neural mechanisms have not been analyzed… (5 年以内に雑誌等で刊行予定のため、非公開。)

In Chapter 2, I established a novel approach for multidisciplinary analyses of Kiss1-Gpr54-1 neuronal system by utilizing a transgenic medaka whose Gpr54-1-expressing neurons are specifically labeled by EGFP. Unlike previous studies, which used fish brains with GFP-labeled ligand-expressing neurons (44,70,71,99,109-111), GFP-labeling of receptor-expressing neurons used in the present study enables identification of the neural system that are potentially regulated by the corresponding ligand-expressing neurons. By this novel technique, I newly identified the neurons producing non-GnRH neuropeptides that can be regulated by Kiss1-Gpr54-1 neuronal system from the results of deep sequencing, histological, and physiological analyses. Among them, distinct populations of neuropeptide B (Npb) neurons in the ventral telencephalon (Vd/Vs/Vp) and ventrolateral preoptic area (vPOA) were shown to express Gpr54-1 and may play various roles in endocrine functions at the pituitary. In addition, neuropeptide Yb (Npyb) and cholecystokinin (Cck) neurons in Vd/Vs/Vp were also found to be Gpr54-1 positive, and they widely spread their axons in rostro-caudal directions in the brain. Similar result has previously been reported in European sea bass, in which Npy neurons expressed *gpr54-2* mRNA (35). This is consistent with the present study, although Kiss2-Gpr54-2 system was suggested to be estrogen insensitive in both medaka and European sea bass (46,81). On the other hand, there has been no report showing the relationships between Kiss1 and Npb neuronal systems to date. Thus, the present study is the first report in vertebrates demonstrating the kisspeptin regulation of Npb neurons. Particularly, using histological and electrophysiological analyses, Gpr54-1/Npb neurons

in vPOA were shown to be hypophysiotropic, and their neuronal activities were facilitated by Kiss1, suggesting Kiss1-Gpr54-1 regulation of endocrine functions via Npb in the pituitary. This is a novel and interesting finding, because there have been only a few hypophysiotropic peptidergic neuronal systems such as GnRH, somatostatin, and several releasing-hormone neurons in vertebrate brains (52,62,112-114), and only a few studies in mammals and no study in teleosts have ever shown that Npb neurons are hypophysiotropic (115,116). Besides, the present study suggested for the first time another novel aspect of Npb neuronal system, that is, the Npb neuronal pathways for the regulation of IT/VT neurons, which may be involved in stress response; very few studies have reported on the function of Npb neurons in non-mammalian species to date (77,79). Moreover, all three neuropeptides, Npy, Cck, and Npb, have been suggested to be involved in various functions such as endocrine regulation, stress response, and feeding regulation in both mammals and teleosts (47,48,50,74,77,78,88-90,116-124). Among them, several pharmacological studies have shown that *in vivo* Npb administration facilitates stress response via increase in ACTH release from the pituitary (48,50,120,125,126). Therefore, it is speculated that hypophysiotropic NPB/W neurons facilitate ACTH release in mammals, although the mechanism is still unclear… (5 年以内に雑誌等で刊行予定のため、非公開。) Taken together, these lines of evidence clearly demonstrate wide range of Kiss1-Gpr54-1 functions via Npyb, Cck, and Npb, which are not directly related to reproductive functions such as gonadal maturation.

Therefore, it is worth noting that I have discovered the existence of neuroendocrine regulatory pathways of Gpr54-1/Npb neurons in vPOA (5 年以内に雑誌等で刊行予定のため、非公開。).

Furthermore, as documented in Chapter 2, I searched for the targets of Gpr54-1/Npb neurons in vPOA and found IT/VT neurons as the strong candidates by observing the characteristic structure of their axonal branches at PPD and PI of the pituitary. These above-mentioned data suggest that Npb can affect IT/VT neurons at intertwined axonal branches. Previous histological studies on IT/VT neurons have suggested that IT/VT neurons are involved in local hormonal secretion in PPD and PI, while they release IT/VT peptides from PN into general circulation for the direct regulation of peripheral tissues (52,62,113,114). Particularly, the local action of VT on ACTH has been suggested by physiological and histological analyses (52,62,113,114).

… (5 年以内に雑誌等で刊行予定のため、非公開。)

In Chapter 3… (5 年以内に雑誌等で刊行予定のため、非公開。)

In summary, the present thesis demonstrated the lines of evidence suggesting the presence of neuronal pathways that consist of Kiss1, Npb, IT/VT neurons and their possible novel functions in medaka, a useful non-mammalian model species. During these 15 years, the search for the kisspeptin functions in non-mammalian species, especially teleosts, has been performed by many researchers. Although it is gradually becoming a general consensus that teleost kisspeptin neuronal system is not involved in the gonadal maturation via the HPG axis regulation, its non-reproductive functions and the mechanisms of these functions still have remained enigmatic in

spite of various studies in various species. This is partly due to technical limitations and difference in animal species used for each study. For the elucidation of functions of the kisspeptin neuronal system in non-mammalian species and for the comparison of such results with those accumulated in mammals to understand the evolution of such functional system, multidisciplinary analyses in a single non-mammalian model animal may be the most effective and powerful approach, as shown in the present study. By taking advantage of medaka, I could examine the kisspeptin neuronal system in detail by comprehensive approaches, at molecular, cellular, and neuronal circuit levels, (5 年以内に雑誌等で刊行予定のため、非公開。), not the exogenous administration of peptides, which has been mostly used in previous studies. Considering the high conservation of above-mentioned neuropeptides in vertebrates, involvement of kisspeptin neurons in the regulation of IT (OT)/ VT (VP) system is most probably a common function of kisspeptin neuronal system that has been conserved throughout vertebrates during evolution, although further comparative studies are necessary (5 年以内に雑誌等で刊行予定のため、非公開。). The present thesis study should be able to give various insights into further understanding of the function and mechanism of kisspeptin neuronal system throughout vertebrates. I believe that my novel experimental approaches, results, and evolutionary insights derived therefrom in my thesis should pave the way towards tackling massive questions on various neuroendocrine mechanisms and that future comparative studies will elucidate their common functions in vertebrates.

Figure 0-2 legend

(5 年以内に雑誌等で刊行予定のため、非公開。)

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