学位論文

Neuroendocrinological studies on central actions of estrogen

(生殖機能制御を中心としたエストロジェン中枢作用の神経内分泌学的研究)

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Abstract

It is now generally accepted in vertebrates that reproductive functions are mainly regulated by a system called the hypothalamo-pituitary-gonadal (HPG) axis. In this regulatory system, gonadal steroids are considered to play important roles. The gonads release sex steroid hormones including estrogen and androgen into the circulation, and the sex steroids, especially estrogen, stimulates the hypothalamus to release hypophysiotropic gonadotropin releasing hormone (called as GnRH1 in most animals). GnRH1 acts on the pituitary and causes the release of the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH released in the general circulation stimulate gonads to induce their maturation or ovulation. Such regulation of reproduction by sex steroids has been well-known as "the sex steroid feedback regulation of reproduction". In this HPG axis regulation system, neurons in the hypothalamus are considered to sense the gonadal states by receiving estrogen from the circulation through estrogen receptor α (ERα).

However, there are many unanswered questions that remain to be addressed. The neural circuitry and mechanisms for the estrogen feedback in the hypothalamus have not yet been clarified. Because the GnRH1 neurons do not express ERα, some hypothalamic estrogen sensitive neurons that express ERα are considered to transmit estrogen feedback signals to the GnRH1 neurons. Recently, genes encoding novel neuropeptide Kisspeptin and its receptor have been identified as responsible genes for hypogonadotropic hypogonadism. Subsequent morphological as well as electrophysiological studies have suggested that Kisspeptin neuron is the target of estrogen feedback signal, and they regulate GnRH1 neurons directly. However, all of these suggestions have been reported in studies using only mammals. Recent studies in

teleosts have suggested that Kisspeptin does not regulate GnRH1 neurons, and the essential role of Kisspeptin in reproductive function should not be considered to be common to all vertebrate species. Therefore, some other neuronal systems that are sensitive to estrogen are considered to play important roles in the HPG axis regulation in non-mammalian vertebrates. With this in mind, I aimed to find mechanisms that are evolutionally conserved throughout vertebrates for the feedback regulation of the HPG axis.

In addition to the HPG axis regulation, estrogen plays crucial roles in many other functions including sexual behaviors. However, the neural circuits for sexual behaviors have not been clarified in detail.

To clarify these general regulatory systems of the reproductive functions including sexual behaviors in vertebrates, I here adopted a challenging and unique approach. Unlike most previous studies that investigated genes of neurotransmitters/neuromodulators that have been suggested to regulate reproductive functions, I addressed this problem by developing a novel tool, *erα*:EGFP transgenic medaka (ERα-Tg medaka). Then I used them to analyze the neural circuitry involved in the coordinated regulation of reproduction and sexual behavior.

In the present thesis, I chose medaka (*Oryzias latipes*) as a model animal, because of its several advantages for the analysis of regulatory systems of the reproductive functions. Because of their completed genome database, medaka allows us easy access to the molecular genetic tools. Furthermore, female medaka spawn daily under breeding condition, and their breeding/nonbreeding states can be easily controlled by changing the day length. Therefore, medaka is an ideal model animal for the neuroendocrinological study of reproductive functions.

In Chapter1, I aimed at detecting the target brain regions for the sex steroid hormones by *in situ* hybridization and anatomically analyzed the distributions of all subtypes of *er* and *ar* mRNA in the brain of female medaka. Results of the Chapter1 suggested some brain regions as the candidate brain nuclei that are involved in the regulations of neural circuits of sexual behaviors or the HPG axis (Vs for regulating motor aspects of various sexual behaviors, and POA for regulating the HPG axis).

In Chapter2, I analyzed direct effects of estrogen on the pituitary. I demonstrated by *in situ* hybridization analysis and suggested that both LH cells and FSH cells co-express ERα. In addition, I applied estrogen to the intact whole pituitary preparations and demonstrated that estrogen directly inhibits the transcription of *fshβ* mRNA, while it does not affect the transcription of *lhβ* mRNA. Therefore, it is suggested that the transcription of *lhβ* is mainly regulated via the HPG axis, while *fshβ* is regulated more strongly by the direct action of gonadal estrogen.

In Chapter3, I analyzed neural circuits of the ERα neurons by generating a transgenic medaka line that expresses *enhanced green fluorescent protein* (EGFP) in their ERα neurons. I analyzed the neural projections and neurotransmitters of the ERα neurons and suggested that POA-glutamatergic and/or GABAergic ERα neurons relay estrogen feedback signals to GnRH1 neurons. I also analyzed the projection of Vs-ERα neurons and found that they project as far as the ventral region of medulla. Taken together with results of the previous studies suggesting involvement of Vs neurons in the regulation of sexual behavior, Vs-ERα neurons are suggested to be involved in the regulation of sexual behaviors in accordance with the levels of estrogen in circulation.

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Abbreviations

AP	area postrema
AR	androgen receptor
BAC	bacterial artificial chromosome
BCIP	5-bromo-4- chloro-3-indoyl-phosphate
BO	olfactory bulb
ca	anterior commissure
cc	central canal
CE	corpus of the cerebellum
dDm	dorsal region of the medial part of dorsal telencephalic area
dDl	dorsal region of Dl
Dl	lateral part of dorsal telencephalic area
DIG	digoxigenin
DM	dorsomedial thalamic nucleus
Dp	posterior part of dorsal telencephalic area
E	epiphysis
E2	17β-estradiol
EGFP	enhanced green fluorescein protein
EmT	eminentia thalami
ER	estrogen receptor
fd	dorsal funiculus
flm	medial longitudinal fasciculus
FSH	follicle stimulating hormone
GABA	gamma amino butyric acid
gad	glutamic acid decarboxylase
GnRH	gonadotropin releasing hormone
GR	corpus glomerulosus
GTH	gonadotropin
HPG axis	hypothalamo-pituitary-gonadal axis
IHC	immunohistochemistry
ISH	*in situ* hybridization

LH	luteinizing hormone
NAT	anterior tuberal nucleus
NBT	4-nitroblue tetrazolium chloride
NCC	commissural nucleus of Cajal
NDTL	diffuse nucleus of torus lateralis
nII	optic nerve
NIXm	nucleus of hypoglossal nerve
NXm	motor nucleus of vagal nerve
NPPv	periventricular posterior nucleus
NRL	nucleus of lateral recess
NVT	ventral tuberal nucleus
OVX	ovariectomy
PBST	phosphate buffered saline with Tween 20
POA	preoptic area
POm	magnocellular preoptic nucleus
POp	parvocellular preoptic nucleus
RFm	medial reticular zone
rPOp	rostral part of POp
SPV	periventriculare stratum
TN	terminal nerve
TO	optic tectum
TS	torus semicircularis
TSc	central nucleus of TS
TSe	external nucleus of TS
TSvl	ventrolateral nucleus of TS
V	ventral telencephalic area
Vd	dorsal part of ventral telencephalic area
ver	rhombencephalic ventricle
vglut	vesicular glutamate transporter
VIIL	facial lobes
VM	ventromedial thalamic nucleus

Vp	postcommissural part of V
Vs	supracommissural part of V
Vv	ventral part of V
XL	vagal lobes

General Introduction

Reproduction is one of the most important functions for all animals. Here, coordinated regulation of gonadal maturation and sexual behaviors by sophisticated neuroendocrine mechanisms are essential for the successful production of offsprings. It is now generally accepted in vertebrates that gonadal maturation is mainly regulated by a system called the hypothalamo-pituitary-gonadal (HPG) axis. In this regulatory system, gonadal steroids are considered to play important roles. The gonads release sex steroid hormones including estrogen and androgen into the circulation, and the sex steroids, especially estrogen, stimulate the hypothalamus to release hypophysiotropic gonadotropin releasing hormone (called as GnRH1 in most animals). GnRH1 acts on the pituitary and causes the release of the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH released in the general circulation stimulate gonads to induce their maturation or ovulation (Childs, 2006). Such regulation of reproduction by sex steroids has been well-known as “the sex steroid feedback regulation of reproduction”. In this HPG axis regulation system, neurons in the hypothalamus are considered to sense the gonadal states by receiving estrogen from the circulation through estrogen receptor (ER)s. It has been reported that there are two groups of estrogen receptors in mammals; ERα and ERβ (Kuiper et al., 1996). Among them, ERα has been suggested by previous studies as an essential factor for the regulation of reproductive functions in mammals (Couse et al., 2003; Dorling et al., 2003).

However, there are many unanswered questions that remain to be addressed. The neural circuitry and mechanisms for the estrogen feedback in the hypothalamus have not yet been clarified. In mice, the GnRH1 neurons do not express ERα (Laflamme et al., 1998; Hrabovszky et al., 2000), and there is no clear evidence that GnRH1 neurons

express ERα in any vertebrates. Therefore, some hypothalamic estrogen sensitive neurons that express ERα are considered to transmit estrogen feedback signals to the GnRH1 neurons. Recently, genes encoding novel neuropeptide Kisspeptin and its receptor have been identified as responsible genes for hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003). Subsequent morphological as well as electrophysiological studies have suggested that Kisspeptin neuron is the target of estrogen feedback signal, and they regulate GnRH1 neurons directly (Messager et al., 2005; Smith et al., 2005; Pielecka-Fortuna et al., 2008). However, all of these suggestions have been reported in studies using only mammals. In non-mammalian species, estrogen sensitivity of Kisspeptin expression has also been suggested in some species of teleosts (Kanda et al., 2008; Kanda et al., 2012). However, recent studies in teleosts have suggested that Kisspeptin does not regulate GnRH1 neurons, and the essential role of Kisspeptin in reproductive function should not be considered to be common to all vertebrate species (Karigo and Oka, 2013). Therefore, some other neuronal systems that are sensitive to estrogen are considered to play important roles in the HPG axis regulation in non-mammalian vertebrates. With this in mind, I aimed to find mechanisms that are evolutionally conserved throughout vertebrates for the feedback regulation of the HPG axis.

In addition to the HPG axis regulation, estrogen plays crucial roles in many other functions including sexual behaviors (Pfaff and Sakuma, 1979; Hull and Dominguez, 2007). For example, in rodents, estrogen is suggested to activate lordosis of female rats (Pfaff and Sakuma, 1979). In teleosts, female guppy, ovariectomy inactivates sexual behaviors, and they can be restored by estrogen treatments (Liley, 1972). Thus, estrogen

plays important roles in the regulations of sexual behaviors. However, the neural circuits for sexual behaviors have not been clarified in detail.

To clarify these general regulatory systems of the reproductive functions including sexual behaviors in vertebrates, I here adopted a challenging and unique approach. Unlike the most previous studies that investigated genes of neurotransmitters/neuromodulators that have been suggested to regulate reproductive functions, I addressed this problem by developing a novel tool, *erα*:EGFP transgenic medaka (ERα-Tg medaka). This has been inspired by the fact that sex steroids exert their effects via binding to the sex steroid receptors expressed in certain neurons. Then I used them to analyze the neural circuitry involved in the coordinated regulation of reproduction and sexual behavior.

In the present thesis, I chose medaka (*Oryzias latipes*) as a model animal, because of its several advantages for the analysis of regulatory systems of the reproductive functions. Because of their completed genome database, medaka allows us easy access to the molecular genetic tools (Okubo et al., 2006). Furthermore, female medaka spawn daily under breeding condition, and their breeding/nonbreeding states can be easily controlled by changing the day length. In addition, medaka show regular one day estrous cyclicity, and a previous study has well documented time of day-dependent changes in the activity or expression of regulatory factors of estrus cycle; GnRH1, LH, and FSH (Karigo et al., 2012). Therefore, medaka is an ideal model animal for the neuroendocrinological study of reproductive functions.

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In Chapter2, I analyzed direct effects of estrogen on the pituitary. I suggested that both LH cells and FSH cells co-express ERα. In addition, I applied estrogen to the intact whole pituitary preparations and demonstrated that estrogen directly inhibits the transcription of *fshβ* mRNA, while it does not affect the transcription of *lhβ* mRNA. Therefore, it is suggested that the transcription of *lhβ* is mainly regulated via the HPG axis, while transcription of *fshβ* is regulated more strongly by the direct action of gonadal estrogen.

In Chapter3, I analyzed neural circuits of the ERα neurons by generating a transgenic medaka line that expresses *enhanced green fluorescent protein* (EGFP) in their ERα neurons. I analyzed the neural projections and neurotransmitters of the ERα neurons and suggested that POA-glutamatergic and/or GABAergic ERα neurons relay estrogen feedback signals to GnRH1 neurons. I also analyzed the projection of Vs-ERα neurons and found that they project as far as the ventral region of medulla. Taken together with results of the previous studies suggesting involvement of Vs neurons in the regulation of sexual behavior, Vs-ERα neurons are suggested to be involved in the regulation of sexual behaviors in accordance with the levels of estrogen in circulation.

Chapter 1

Anatomical distribution of sex steroid hormone receptors in the brain of female medaka

Abstract

Estrogen and androgen play crucial roles in coordinating reproductive functions through estrogen receptors (ER) and androgen receptors (AR), respectively. These receptors are supposed to be important for the regulation of hypothalamo-pituitary-gonadal (HPG) axis. Despite their biological importance, the distribution of sex steroid receptors has not been fully analyzed anatomically in the teleost brain. The teleosts show many characteristic features, which enable unique approaches toward understanding regulatory mechanisms of reproductive functions. Medaka serves as a good model system for studying the mechanisms by which steroid receptor-mediated systems are regulated, because1) their breeding conditions can be easily manipulated, 2) I can take advantage of the genome database, and 3) molecular genetic tools, such as transgenic techniques, are applicable. I analyzed the distribution of *erα*, *erβ1*, *erβ2*, *arα*, and *arβ* mRNA by *in situ* hybridization in the brain of female medaka. I found that all subtypes of ERs and ARs were expressed in the following nuclei, dorsal part of ventral telencephalic area (Vd), supracommissural part of ventral telencephalic area (Vs), postcommissural part of ventral telencephalic area (Vp), preoptic area (POA), and nucleus ventralis tuberis (NVT). These regions are known to be involved in the regulation of sexual behavior (Vd, Vs, Vp) or HPG axis (POA, NVT). These ER and/or AR expressing neurons may regulate sexual behavior or HPG axis according to their axonal projections. Future analysis should be targeted to the neurons described in the present Chapter to extend our understanding of the central regulatory mechanisms of reproduction.

Introduction

Actions of estrogen and androgen, the major sex steroid hormones in vertebrates, are mediated through their receptors; estrogen receptor (ER) and androgen receptor (AR). These sex steroid hormone receptors belong to the members of the nuclear receptor superfamily (Escriva et al., 2000). Both ER and AR function as transcription factors when activated by the respective sex steroids and play crucial roles in coordinating reproductive functions as well as many other functions, including sexual behavior and learning in accordance with the changes in reproductive stages (Hull and Dominguez, 2007; Galea et al., 2008; Luine, 2008). Especially, there is no doubt that sex steroid hormone receptors are essential for the well-known positive and negative feedback regulation of the hypothalamo-pituitary-gonadal (HPG) axis (Vadakkadath Meethal and Atwood, 2005). Previous studies, which focused on the function of ER or AR, provided evidence for the mechanism of HPG axis regulation. Studies using ERα and ERβ knockout mice strongly suggested that ERα may be more important for reproduction in mice (Couse et al., 2003; Doring et al., 2003). On the other hand, it has also been shown that gonadotropin-releasing hormone (GnRH) neurons, the final common pathway for reproductive regulation, do not express ERα in mice and several mammals (Laflamme et al., 1998; Hrabovszky et al., 2000). There exist many questions that still remain to be clarified concerning not only the steroid feedback mechanisms but also the possible non-reproductive functions of sex steroid hormones.

Teleost fish show many characteristic features, such as sex reversal, characteristic sexual behaviors, and seasonal change of reproductive condition. These features enable good opportunities for unique approaches toward the understanding of the central mechanisms that underlie reproductive functions. Especially, medaka *Oryzias latipes*

serves as a good model system for studying the mechanisms by which HPG axis and other steroid receptor-mediated systems are regulated, because medaka possesses all three functionally and morphologically differentiated GnRH neuronal groups, for which GFP transgenic animals are now available (Karigo et al., 2012; Okubo et al., 2006) Moreover, such molecular genetic tools are applicable rather easily in medaka because of its fully-equipped genome database. In addition, female medaka spawns daily under the breeding condition, and their breeding and nonbreeding states are controllable by changing daily light cycle. Besides, they are rather easily amenable to experimental surgeries such as ovariectomy, which will allow us to study functions of the sex steroid-sensitive neurons. Thus, the female medaka is an ideal model for the study of feedback mechanism of the HPG axis. For a detailed analysis of the central roles of estrogens and androgens during the feedback regulation, the information about distribution of ER- and AR-expressing neurons in the brain is critically important. However, in spite of the interesting biological features, there are only a small number of studies that report the distribution of sex steroid receptors in the brain of teleosts. Either ER or AR localization has been studied in the oyster toadfish (Fine et al., 1996), seabass (Muriach et al., 2008) , or Atlantic croaker (Hawkins et al., 2005). Both ER and AR expression have been reported in a few teleosts including the goldfish (Kim et al., 1978; Gelinas and Callard, 1997), zebrafish (Menuet et al., 2002; Gorelick et al., 2008), midshipman fish (Forlano et al., 2005; Forlano et al., 2010), and African cichlid fish (Harbott et al., 2007; Munchrath and Hofmann, 2010), but some of these studies did not distinguish ER or AR subtypes. In the present Chapter, I scrutinized the distribution of *erα*, *erβ1*, *erβ2*, *arα*, and *arβ* mRNA separately by using *in situ* hybridization specific for each type of receptor in the whole brain of female medaka. *In situ* hybridization is an effective

technique, which enables specific detection of the mRNA with low levels of expression, and it enables the distinction of subtypes of ERs and ARs. Morphological studies of the expression of sex steroid hormone receptors should provide important information toward the understandings of the regulatory mechanisms of the HPG axis and other important functions of sex steroids by using medaka as a model system.

Materials and methods

Animals

Female d-rR medaka (*Oryzias latipes*) were maintained under a 14 h light and 10 h dark photoperiod at water temperature of 27°C. Fish were fed twice or three times a day with live brine shrimp and flake food. All animals were maintained and used in accordance with the guidelines of the University of Tokyo for the Use and Care of Experimental Animals.

In situ hybridization analysis

I used 0.27 to 0.37 g mature female medaka (more than 3 months after hatching) that spawned eggs for at least 3 consecutive days. Medaka were deeply anesthetized with MS-222 (Sigma, St. Louis, MO). In most cases, brains were dissected and immersed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for several hours at 4°C for fixation, and immersed in PBS containing 30% sucrose for a few days. For detection of *er* or *ar* mRNA with lower expression level in the hindbrain and spinal cord, medaka were perfused transcardially with 4% PFA in PBS to increase the preservation of RNA, and decapitated. The brains were further fixed with 4% PFA in PBS again for 10 min at 4°C, and immersed in PBS containing 30% sucrose until

they sank to the bottom of the tube. Transcardial perfusion improved the sensitivity of *in situ* hybridization in the hindbrain and the spinal cord. As a result, positive signals for *er* and *ar* mRNA were detected in the hindbrain of transcardially perfused brains, but not detected in the hindbrain of samples that is fixed without perfusion. However, in another regions, the results were completely the same between the specimens with and without perfusion fixation. After the end of the sucrose treatment, the brains were then embedded in 5% agarose (Type IX-A, Sigma) containing 20% sucrose in PBS, frozen in n-hexane at around -60°C, sectioned frontally at 20 μm on a cryostat at -20°C, and mounted onto MAS-GP-coated glass slides (Matsunami, Osaka, Japan). To detect *er* and *ar* mRNA, I prepared a specific digoxigenin (DIG)-labeled riboprobe. The template used to make the *erα* probe was 1078 bp in length (DDBJ/EMBL/ GenBank accession no. AB033491); the *erβ1* probe was 1202 bp in length (GenBank accession no. AB070901); the *erβ2* probe was 1210 bp in length (Genbank accession no. AB428449); the *arα* probe was 1181 bp in length (GenBank accession no. AB252233) (Ogino et al., 2009); the *arβ* probe was 1015 bp in length (GenBank accession no. EU100398). The probes for these genes were synthesized from the medaka brain using a labeling kit (Roche Molecular Biochemicals GmbH, Mannheim, Germany) (See Table1 for primer information). All the sections were washed with PBS, treated with 1μg/ml protease K (Takara, Shiga, Japan) for 15 min at 37°C, postfixed with 4% paraformaldehyde in PBS for 15 min, treated with 2 mg/ml glycine in PBS for 10 min, and incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Then, the sections were prehybridized at 58°C for at least 30 min in hybridization buffer containing 50% formamide, 3X saline sodium citrate (SSC), 0.12 M phosphate buffer (pH 7.4), 1X Denhardt's solution (Sigma), 125 μg/ml tRNA, 0.1 mg/ml calf thymus DNA

(invitrogen, California, United States), and10% dextran sulfate (Sigma). The slides were incubated at 58°C overnight in the same solution containing 100 ng/ml denatured riboprobe. After hybridization, the sections were washed twice with 50 % formamide and 2X SSC for 15 min each at 58°C. The sections were then washed with 2X SSC twice, followed by 0.5X SSC twice for 15 min each at 58°C. The slides were immersed in DIG-1 (0.1 M Tris-HCl, 0.16 M NaCl, and 0.1% Tween 20) for 5 min, 1.5% blocking reagent with DIG-1 for 30 min, and DIG-1 for 15 min, and then incubated with an alkaline phosphatase-conjugated anti-DIG antibody (diluted 1:3000 with DIG-1, Roche) for at least 1h. The sections were washed with DIG-1 twice for 15 min each, and DIG-3 (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl; 0.05 M $MgCl_2$) for 3 min. The sections were then treated with a chromogenic substrate containing 337 μg/ml 4-nitroblue tetrazolium chloride (NBT) and 175 μg/ml 5-bromo-4- chloro-3-indoyl-phosphate (BCIP) in DIG-3 until a visible signal was detected. The sections were immersed in a reaction stop solution (10 mM Tris-HCl, pH8.0; 1mM EDTA, pH8.0) to stop the chromogenic reaction. They were then dehydrated, cleared, coverslipped, and observed under a light microscopy. 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).

Double-label *in situ* hybridization for *erα* mRNA and *gnrh3* mRNA

Because our preliminary *in situ* hybridization of *erα* mRNA strongly suggested that the terminal nerve (TN)-GnRH neurons express ERα, I performed a double-label *in situ* hybridization using a mixture of DIG-labeled *erα* and fluorescein-labeled *gnrh3* probes,

as previously reported (Akazome et al., 2011). All the procedures up to the blocking step were performed as described above (brains were fixed by transcardial perfusion). Then, the sections were incubated with a horseradish peroxidase-conjugated anti-fluorescein antibody (diluted 1:500 with DIG-1, PerkinElmer, Foster City, CA) for at least 1 h. Sections were washed twice with DIG-1 for 5 min, incubated with Biotinyl Tyramide Amplification kit (TSA, NEL700A, PerkinElmer) (diluted 1:50 in dilution buffer; PerkinElmer) for 30 min, washed with DIG-1 twice for 5 min each, and incubated with avidin biotin complex reagents (1% A solution and 1% B solution in DIG-1; Vector, Burlingame, CA) for 1 h. The sections were washed twice with DIG-1 for 5 min, incubated with Alexa Fluor 488-conjugated streptavidin (diluted 1:500 with DIG-1; Invitrogen, Carlsbad, CA) and alkaline phosphatase-conjugated anti-DIG antibody (diluted 1:1000 with DIG-1, Roche) for 2 h. Then the sections were washed twice with DIG-1 for 5 min. After detection of *gnrh3* signals, the sections were incubated with DIG-3 for 5 min. Alkaline phosphatase activity was detected using an 2-hydroxy-3-naphatonic acid-2'-phenylanilide phosphate (HNPP) fluorescence detection kit (Roche) according to the manufacturer's instructions. The incubation for this substrate was carried out until visible signals were detected and was stopped by washing in PBS containing 0.5 mM EDTA. The sections were then coverslipped with CC/Mount (Diagnostic BioSystems, Pleasanton, CA).

Nissl staining

I performed Nissl staining of female medaka brain. The procedures up to the sectioning step are as described above. The sections were stained by 0.1% cresyl violet solution. Then, they were dehydrated, cleared, and coverslipped.

Photomicroscopy

Bright field optics was used to visualize samples. Photographs were taken with a digital camera (DFC310FX; Leica Microsystems, Wetzlar, Germany) attached to a microscope (DM5000B; Leica Microsystems, Wetzlar, Germany). Fluorescent sections were examined using a confocal laser-scanning microscope (LSM-710; Carl Zeiss, Oberkochen, Germany).

Images were compiled and brightness- and contrast- enhanced in Adobe Photoshop (San Jose, CA).

Results

An atlas of the female medaka brain was drawn from the Nissl stained sections as shown in Figure1. Schematic illustrations (Fig. 1 A-J) and representative photographs (Fig. 2 A-H) indicate the brain regions in which *er* and/or *ar* mRNA positive neurons were mainly located. In the present Chaper, I used the term rPOp as representing the rostral part of the preoptic area (POA).

mRNAs for both *er*s and *ar*s of all subtypes were expressed by the neurons in the following structures; dorsal part of ventral telencephalic area (Vd), supracommissural part of ventral telencephalic area (Vs), postcommissural part of ventral telencephalic area (Vp), rPOp, magnocellular preoptic nucleus (POm), periventricular posterior nucleus (NPPv), and ventral tuberal nucleus (NVT). In addition, all subtypes of *er*s and *ar*s mRNA were diffused along the rhombencephalic ventricle (ver). On the other hand, not all but several subtypes of *er* or *ar* mRNA were differentially expressed by the neurons in some nuclei; lateral part of dorsal telencephalic area (Dl) (*arα*), olfactory

bulb (BO) (*erα* and *arα*), ventral part of ventral telencephalic area (Vv) (*erβ1*, *arα* and *arβ*), eminentia thalami (EmT) (*arα* and *arβ*), ventromedial thalamic nucleus (VM) (*erβ2*), dorsomedial thalamic nucleus (DM) (*arα* and *arβ*), optic tectum (TO) (*arα* and *arβ*), torus semicircularis (TS) (*arα*), dorsal to flm in the mesencephalon, (*erα* and *arα*), and medial *reticular zone* (RFm) (*erα*, *arα* and *arβ*). After careful and thorough examination of the entire brain, I found that neither *erα* mRNA nor *arα* mRNA was expressed in the nucleus of solitary tract, which contains sex steroid receptors in the brain of rodents (Simerly et al., 1990; Shughrue et al., 1997) (Fig. 3). The distribution of neurons expressing mRNAs for each *er* and *ar* subtype in the female medaka brain is summarized in table (Table 2) and schematic brain atlas. For each of the *er*/*ar* subtype, schematic drawings as well as photomicrographs of corresponding areas of representative sections are shown in each figure (Figs. 4-5, 7-12, 14-15). All the control slides that were hybridized with respective sense probes for *in situ* hybridization showed no specific signals, warranting the specificity of neurons labeled with all the antisense probes. The following sections describe the distribution of neurons expressing different types of receptor mRNAs.

Estrogen receptor alpha

erα mRNA-expressing neurons (ERα neurons) were distributed in some structures of the telencephalon, diencephalon, mesencephalon, and hindbrain (Figs.4 and 5). Most rostrally, the ERα neurons were found in the terminal nerve (TN) (Fig. 4 A, Fig. 5 A). Their cell size and localization suggest that ERα is expressed in the terminal nerve (TN)-GnRH neurons in the boundary of BO and Vv. To examine whether ERα is expressed in TN-GnRH neurons or not, I performed a double-label *in situ* hybridization

for *erα* mRNA and *gnrh3* mRNA, which is produced by TN-GnRH neurons. The results indicated that *gnrh3* mRNA-positive signal co-localized with *erα* mRNA signal (Fig. 6). Thus, it was shown that the TN-GnRH neurons express ERα.

The ERα neurons were also distributed in the Vd, Vs, and Vp (Fig. 4 B-D, Fig. 5 B). Judging from the apparent density of chromogenic precipitations in the cells as well as the time for chromogenic precipitations to reach visible densities during the alkaline phosphatase reactions, the strongest expression of *erα* mRNA in the brain was localized in the rostral part of POp (rPOp) and POm (Fig. 4 C, D, E, Fig. 5 C and D), whereas they were not distributed in the caudal part of POp that lies ventral to the POm (Fig. 4 E). In the diencephalon, the ERα neurons were found in the NVT (Fig. 4 F-G, Fig. 5 E) and NPPv (Fig. 4 F-G). ERα neurons were also found in the area dorsal to flm in the mesencephalon (Fig. 4 H). In addition, ERα neurons were found along ver (Fig. 4 I-J, Fig. 5 F-I) and in RFm (Fig. 4 J) in the hindbrain. Higher magnification slides of Nissl stained section (Fig. 5 H) and ERα-expressing cells (Fig. 5 I, arrow heads) in the region that is close to ver suggest that most of these ERα positive small cells are neurons.

Estrogen receptor beta1 and beta2

erβ1 and *β2* mRNA-expressing neurons (ERβ1 and ERβ2 neurons, respectively) were distributed in some structures of the telencephalon, diencephalon, and hindbrain (Figs. 7-10). The distribution of ERβ1 and ERβ2 neurons largely overlapped with that of ERα neurons. However, in the telencephalon, ERβ1 neurons were distributed in Vv (Fig. 7 A, Fig. 8 A), where ERβ2 positive cell were not found. Similar to ERα neurons, both ERβ1 and ERβ2 neurons were distributed in Vd, Vs, and Vp (Fig. 7 A-C, Fig. 8 B, Fig. 9 A-C, Fig. 10 A). In the POm and rPOp, both ERβ1 and ERβ2 neurons were

extensively distributed (Fig. 7 B-D, Fig. 8 C-D, Fig. 9 B-D, Fig. 10 B-C), whereas there were few positive signals in caudal part of POp, which is ventral to POm (Fig. 7 D, Fig. 9 D). The distribution of ERβ1 and ERβ2 neurons in the diencephalon also overlapped with that of ERα neurons. Both ERβ1 and ERβ2 neurons were observed in NVT (Fig. 7 E-F, Fig. 8 E, Fig. 9 E-F, Fig. 10 D) and NPPv (Fig. 7 E-F, Fig. 8 F, Fig. 9 E-F, Fig. 10 E). ERβ2 but not ERβ1 neurons were localized in VM (Fig. 9 E). In the hindbrain, both ERβ1 and ERβ2 neurons were distributed along the ver (Fig. 7 G-H, Fig. 8 G-H, Fig. 10 F).

Androgen receptor alpha

arα mRNA-expressing neurons (ARα neurons) were distributed in some structures of the telencephalon, diencephalon, mesencephalon, and hindbrain (Fig. 11, Fig. 12). Some ARα neurons showed similar distribution pattern to the ERα, ERβ1 or ERβ2 neurons. Most rostrally, the ARα neurons were found in Dl (Fig. 11 A), where no ER neurons were detected, and BO (Fig. 11 A, Fig. 12 A). In BO, *arα* was localized in the granule cell layer. ARα neurons were also observed in Vv (Fig. 11 B, Fig. 12 B). In addition, ARα neurons were found in Vd, Vs, Vp (Fig. 11 B-D, Fig. 12 C), POm (Fig. 11 D-E, Fig. 12 E) and rPOp (Fig. 11 C, Fig. 12 D). In these areas, ARα neurons showed similar distribution pattern to ER neurons. ARα neurons were widely distributed in the rPOp. In the diencephalon, ARα neurons were distributed in EmT (Fig. 11 E, Fig. 12 E), DM (Fig. 11 F), NVT (Fig. 11 F-G, Fig. 12 F) and NPPv (Fig. 11 F-G). In the mesencephalon, periventriculare stratum (SPV) of TO (Fig. 11 F-G), TS (Fig. 11 G), and the area dorsal to flm, the ARα neurons were detected by *in situ* hybridization, but no overlap with *er* expression was shown in these areas. TS is known to be composed of

some different subdivisions. For instance, TS of goldfish is composed of three different subdivisions; ventrolateral nucleus (TSvl), central nucleus (TSc), and external toral nucleus (TSe) (Yamamoto et al., 2010). In the present Chapter, Nissl sections showed that TS of medaka contain three different subdivisions (Fig. 13 A, B). Although further analysis is needed to describe the functional difference between these three subdivisions in medaka, I found *arα* mRNA in the lateral area of each three different subdivision of TS by Nissl staining and *in situ* hybridization (Fig. 13 C-D). In the hindbrain, ARα neurons were distributed along the ver (Fig. 11 I-J, Fig. 12 J) and RFm (Fig. 11 J, Fig. 12 H).

Androgen receptor beta

In general, *arβ* mRNA-expressing neurons (ARβ neurons) were distributed in the similar areas as that of *arα* mRNA. The ARβ neurons were observed in Vv (Fig. 14 A), Vd, Vs, Vp (Fig. 14 A-C, Fig. 15 A), POm (Fig. 14 C-D, Fig. 15 C) and rPOp (Fig. 14 B, Fig. 15 B). In these areas, the distribution pattern was similar to that of ARα neurons. However, ARβ neurons were not distributed in Dl and BO, where *arα* was expressed. In the diencephalon and mesencephalon, ARβ neurons were distributed in EmT (Fig. 14 D), DM (Fig. 14 E), SPV of TO (Fig. 14 E-F), NVT (Fig. 14 E-F, Fig. 15 D), and NPPv (Fig. 14 E-F, Fig. 15 E). In the hindbrain, ARβ neurons were distributed along the ver and in RFm (Figs. 14 G-H).

Discussion

Distribution of ERs and ARs in the brain compared with other teleosts

In the present Chapter, I analyzed the distribution of ERs and ARs in the brain of female medaka. Here I compared the results with previous *in situ* hybridization studies for other teleosts; African cichlid fish *Astatotilapia burtoni* (ERα, ERβ1, ERβ2, ARα and ARβ (Harbott et al., 2007; Munchrath and Hofmann, 2010), plainfin midshipman *Porichthys notatus* (ERα and AR) (Forlano et al., 2005; Forlano et al., 2010), and zebrafish *Danio rerio* (ERα, ERβ1, ERβ2 and AR) (Menuet et al., 2002; Gorelick et al., 2008).

Olfactory bulb (BO)

I found that BO contains *arα* mRNA. The study for African cichlid fish showed that all subtypes of *er* mRNA and *ar* mRNA are expressed in the granule cell layer of BO (Munchrath and Hofmann, 2010). In medaka, *arα* mRNA was distributed in the granule cell layer. On the other hand, *in situ* hybridization study indicated that not *ar* mRNA but *erα* mRNA is expressed in BO of midshipman fish (Forlano et al., 2005; Forlano et al., 2010). In zebrafish, neither *er* nor *ar* mRNA was detected in BO (Gorelick et al., 2008; Menuet et al., 2002). These studies indicate that expression of ER and AR in BO is different in each species. This species difference may suggest the difference in the participation of sex steroids in the central processing of the olfaction among species. Although the effects of steroids have not been analyzed precisely, previous study has suggested that olfactory responses to prostaglandins showed differences between species (Kitamura et al., 1994). Therefore, the elucidation of the functional significance of presence or absence of AR or ER may be interesting for the future studies. In addition, I found that *erα* mRNA was expressed in the TN-GnRH neurons, which reside in the boundary of BO and Vv. Previous studies for other teleosts have not reported the

expression of ER in the TN-GnRH neurons. I will discuss the function of ERα in the TN-GnRH neurons below.

Telencephalon

In the telencephalon of medaka, subtypes of both ER and AR were mainly distributed in the ventral area. *er* and/or *ar* mRNA expressing neuron was not found in the dorsal telencephalic area except *arα* in Dl. The distribution of *er* mRNA and *ar* mRNA in the telencephalon of zebrafish was similar to medaka. *ar* mRNA was found in Dl, but there is no report that *er* mRNA was found in the dorsal telencephalic area of zebrafish (Gorelick et al., 2008; Menuet et al., 2002). In the telencephalon of African cichlid fish, all subtypes of *er* mRNA and *ar* mRNA were widely distributed in the dorsal area (Harbott et al., 2007; Munchrath and Hofmann, 2010). The dorsal telencephalic area of the midshipman fish also contained *erα* mRNA and *ar* mRNA (Forlano et al., 2005; Forlano et al., 2010). The present Chapter and the previous studies indicated that these teleosts express *er* mRNA and *ar* mRNA abundantly in the ventral telencephalic area in common. Both *er* and *ar* mRNA were expressed in Vs of medaka, African cichlid fish, and midshipman fish. In addition, POA were abundant in *er* mRNA and *ar* mRNA in all these species. Such expression patterns of sex steroid hormone receptors in POA may be conserved throughout teleosts.

Diencephalon

In medaka, all subtypes of *er* mRNA and *ar* mRNA were expressed in NPPv and NVT. Especially, I demonstrated that *er* mRNA positive cells and *ar* mRNA positive cells were localized in NVT and they expressed mRNA at high levels in medaka.

Previous studies also reported on their prominent expression in the ventral part of the hypothalamus (Forlano et al., 2005; Forlano et al., 2010; Gorelick et al., 2008; Menuet et al., 2002), although the African cichlid fish showed much broader expression (Harbott et al., 2007; Munchrath and Hofmann, 2010).

Mesencephalon and hindbrain

In the mesencephalon, both *arα* mRNA and *arβ* mRNA were found in TO, and only *arα* mRNA was found in TS of medaka. *er* mRNA was not found in these areas. Studies in the zebrafish showed that TO contains *ar* mRNA, but it does not contain *er* mRNA (Gorelick et al., 2008; Menuet et al., 2002), and also showed that neither *er* mRNA nor *ar* mRNA is expressed in TS. On the other hand, previous studies in the African cichlid fish (Harbott et al., 2007; Munchrath and Hofmann, 2010) and midshipman fish (Forlano et al., 2005; Forlano et al., 2010) showed that both *er* mRNA and *ar* mRNA are localized in these areas; TO and TS.

In the hindbrain, all subtypes of *er* mRNA and *ar* mRNA were distributed in RFm, and also distributed along the ver in medaka brain. Interestingly, distribution patterns of both *er* and *ar* mRNAs in ver of medaka brain were similar to that of AR of the midshipman fish (Forlano et al., 2010). On the other hand, neither *er* mRNA nor *ar* mRNA was found in RFm in zebrafish, African cichlid and midshipman fish. The function of ER and AR in the hindbrain of teleosts has not been analyzed enough, except for midshipman fish vocal regulation in the breeding season. *ar* mRNA are localized in the vocal pattern generator region of the medulla oblongata of the midshipman fish, which participates in the auditory processing in a steroid-dependent manner (Bass, 2008).

In rodents, in contrast, the expressions of both ER and AR in the hindbrain and spinal cord were more widely distributed, including nucleus of the solitary tract, and these sex steroid receptors are suggested to be involved in the regulation of sexual behavior, homeostasis or feeding (Simerly et al., 1990; Shughrue et al., 1997; Merchenthaler et al., 2004; Vanderhorst et al., 2005). The results of the present and previous studies in teleosts may suggest that the estrogen and/or androgen receptors in these caudal brain regions have less diverse functions compared to mammals.

As compared in this section, the present Chapter and the previous results in some teleost fishes suggest that *er* mRNA and *ar* mRNA show similar expression patterns in some brain regions. Especially, the POA and the ventral part of hypothalamus contain both *er* mRNA and *ar* mRNA abundantly, regardless of species. In some regions, the expression of the receptors differed depending on the fish species. Especially, the African cichlid fish has been reported to express both *er* mRNA and *ar* mRNA much more widely throughout telencephalon to diencephalon, compared to the other teleost species.

Functional significance of ERs and ARs in the brain

I found that neurons expressing mRNAs for all subtypes of both *er*s and *ar*s were distributed in the following nuclei; Vd, Vs, Vp, rPOp, POm, NPPv, and NVT. The results suggest that the estrogen- or androgen- sensitive neurons are abundant in these areas, and they are probably involved in the regulation of various functions that are under the influence of the respective sex steroids. Several studies have indicated that many of these brain regions are involved in the functions related to reproduction; sexual

behavior, spawning, or HPG axis regulation. Thus, I will discuss below possible involvement of these steroid hormone receptors in these functions.

Olfactory bulb (BO), Optic tectum (TO), Torus semicircularis (TS)

Among the brain regions that are related to sensory information processing, AR but not ER was expressed in TO (ARα and ARβ), TS (ARα) and granule cell layer of BO (ARα). It may suggest that androgen, rather than estrogen, is more closely involved in the sensory processing. TS is supposed to receive the secondary and tertiary somatosensory, auditory and lateral line inputs (McCormick and Hernandez, 1996; Yamamoto et al., 2010). TO is suggested to integrate retinal inputs with nonvisual multimodal sensory inputs and relay them to different brain regions (Kinoshita and Ito, 2006; Kinoshita et al., 2006). BO receives external chemical cues and transmits it to different areas of the brain. Especially, the morphology and physiology of teleost BO has been rather well studied (Kawai et al., 2009). Thus, the function of ER and AR in BO will be discussed here.

In the present Chapter, I showed that *arα* mRNA was expressed in the internal cell layer of BO in which the interneurons equivalent to the granule cells of the mammalian olfactory bulb are distributed. Previous studies in mice suggested that testosterone treatment increases neuronal firing in the BO in response to female urine odors (Pfaff and Pfaffmann, 1969). Other previous studies have reported expression of all subtypes of ER and AR in BO of the African cichlid fish, *Astatotilapia burtoni* (Maruska and Fernald, 2010; Munchrath and Hofmann, 2010). In addition, studies using the African cichlid fish indicated that mRNA levels of both *er* and *ar* in BO change according to the social status or reproductive condition (Maruska and Fernald, 2010). Thus, it is possible

that sex steroid receptors are involved in the modulation of olfactory response and that change in receptor expression is related to the reproductive and/or the social status of medaka. In medaka, among subtypes of *er* and *ar*, only *arα* was expressed in internal cell layer of BO. Therefore, only ARα can participate in such functions. Given that sex steroid receptors are expressed in BO of mammals and teleots (Guo et al., 2001; Portillo et al., 2006; Maruska and Fernald, 2010; Munchrath and Hofmann, 2010), involvement of sex steroids in such regulatory mechanism may be conserved among these species. It has also been reported that TN-GnRH neurons are often localized in close association with the BO across vertebrates including teleosts (Amano et al., 1991; Oka and Matsushima, 1993; Kawai et al., 2010) , amphibians (D'Aniello et al., 1995), birds (Teruyama and Beck, 2000) , and mammals (Kim et al., 1999).

TN-GnRH neurons have been supposed to regulate motivational state for the sexual behavior (Yamamoto et al., 1997), and their physiological activity and morphological features have been well characterized (Kawai et al., 2009; Oka, 2009; 2010). However, it is still unclear whether the sex steroid hormones are involved in the regulation of TN-GnRH neurons. The results of the present Chapter strongly suggest a direct regulation of estrogen on TN-GnRH neurons, because TN-GnRH neurons were clearly positive for *erα* mRNA (Fig. 6). Thus, it is possible that medaka TN-GnRH neurons change their firing activities and/or other properties, which may result in the changes in the motivational state for behaviors in accordance with the breeding conditions. The present new finding that TN-GnRH neurons express ERα is consistent with several previous studies indicating estrogen effects on the TN-GnRH neurons. For example, immunohistochemical study reported that ovariectomy (OVX) and the estrogen replacement alters TN-GnRH fiber density in BO of female *Xenopus laevis* (Wirsig-

Wiechmann and Lee, 1999). In addition, removal of estrogen reduces spiny processes and synaptic inputs in the hypothalamic GnRH neurons in mammals (Witkin et al., 1991; Witkin, 1996). Therefore, it is very interesting future topic to analyze estrogen dependent ultrastructural changes in TN-GnRH neurons to evaluate the hormonal regulation of this neural system. The dwarf gourami, *Colisa lalia* will serve as a good model system for such studies, because the primary culture of TN-GnRH neuron is already established, and it is possible to analyze firing activities of single TN-GnRH neuron under diverse sex steroid milieu (Abe and Oka, 2009).

Ventral telencephalic area

In the present Chapter, all the subtypes of ER and AR were demonstrated to be highly expressed in Vd, Vs, Vp. Previous studies have also suggested the expression of ER and AR in these brain areas of several teleost species (Fine et al., 1996; Gelinas and Callard, 1997; Menuet et al., 2002; Forlano et al., 2005; Hawkins et al., 2005; Muriach et al., 2008; Munchrath and Hofmann, 2010). Thus far, lesion and stimulation studies in teleosts have suggested that Vv, Vd, Vs, and Vp are involved in the central regulation of sexual behavior. In the hime salmon brain, electrical stimulation of these specific brain regions immediately evoked sexual behaviors, suggesting that these regions may function as a part of the neural circuit for the sexual behavior, especially in its motor aspects (Satou et al., 1984). However, there has been no evidence that estrogen or androgen solely triggers sexual behaviors in any teleost fish. Regulation of sexual behavior is well documented in the female goldfish (Munakata and Kobayashi, 2010), whose sexual behavior is induced by prostaglandin F2α (PGF2α). Although androgens do not trigger sexual behaviors, they could be important for induction of sexual

behaviors in the male goldfish. In several teleost species, it has been reported that castrated male fish does not show sexual behaviors, and androgen administration restores their sexual behaviors. In addition, it has also been reported that administration of androgen enables sexually immature males and females to exhibit male-typical sexual behavior (Smith, 1969; Kindler et al., 1991; Stacey and Kobayashi, 1996). These previous studies suggested that gonadal androgen may modulate sexual behaviors. On the other hand, ovariectomized females exhibit sexual behavior after the administration of PGF2α. Thus, estrogen may not regulate sexual behavior directly. This result does not eliminate a possible participation of estrogen in the expression of sexual behavior in some aspects. Precise morphological and physiological analyses for the nuclei Vv, Vd, Vs, and Vp are necessary to check whether estrogen is involved in the regulation of sexual behaviors.

Magnocellular preoptic nucleus (POm)

In the present Chapter, expression of all subtypes of *er* and *ar* mRNA in POm was demonstrated. Previous studies have shown that arginine vasotocin (AVT) neurons are localized in this region in several teleosts (Van den Dungen et al., 1982; Larson et al., 2006; Greenwood et al., 2008). It has been suggested that AVT is involved in aggressive behavior, social dominance, or reproductive behaviors in vertebrates (Semsar et al., 2001; Salek et al., 2002; Santangelo and Bass, 2006), although it appears to be still controversial. Immunohistochemical study of medaka brain has shown that vasotocin (VT) and/or isotocin (IT), a counterpart of arginine vasopressin (AVP) and oxytocin of mammals respectively, were expressed in POm region (Ohya and Hayashi, 2006). Ohya and Hayashi indicated that the number of medaka POm-VT/IT immunoreactive neurons

decreased after spawning. In addition, in the pre-spawning medaka, VT/IT-immunoreactive neurons were larger than those in the post-spawning females. These results suggest that VT/IT neurons are somehow involved in reproduction. A previous study in mice showed that some ERβ immunoreactive neurons co-express AVP in the hypothalamus (Alves et al., 1998). In addition, *in vitro* study has been shown that AVP gene has estrogen response element (Shapiro et al., 2000). These studies suggest that estrogen could be involved in the regulation of AVP. Although the influence of estrogen on AVT neurons in medaka has not been clarified yet, subpopulation of medaka POm may participate in the regulation of spawning by changing VT and/or IT secretion level in a sex steroids-dependent manner.

Nucleus ventralis tuberis (NVT) and preoptic area (POA)

POA (called rPOp in the present Chapter) and NVT regions are suggested to be involved in the regulation of HPG axis. Previously, it has been suggested that most NVT-ER neurons express Kiss1 (Mitani et al., 2010). The Kiss1 neurons are now generally considered as an essential regulator of HPG axis in mammals, because several studies have indicated that Kiss1 neurons exhibit steroid sensitivity, and that Kiss1 facilitates the activity of GnRH neurons (Irwig et al., 2004; Messager et al., 2005; Smith et al., 2005). However, in non-mammalian species, previous morphological studies suggested that NVT-Kiss1/ER neurons do not regulate GnRH1 neuron directly, because they do not express GPR54 in several teleost (Kanda et al., 2013). Furthermore, recent gene knock out studies in zebrafish (Tang et al., 2014) suggested that NVT-Kiss1 neurons are not essential for reproduction. From these results, ERs and ARs in NVT region are suggested not to be involved in the regulation of HPG axis in non-

mammalian species including teleosts. On the other hand, POA region is known to contain GnRH1 neurons or dopamine neurons, which are suggested to regulate reproductive functions. Dopamine neurons are suggested to project to the pituitary and inhibit GnRH1-mediated LH/FSH release. Besides, in teleosts, anatomical study suggested that both GnRH1 and dopamine neurons in POA directly project their axons to the pituitary, and some of them may partly be involved in the regulation of gonadotropes (Karigo et al., 2012; Singh et al., 2012). These results suggest a direct role of POA in the regulation of pituitary functions. Results of the present Chapter and previous study (Hiraki et al., 2012) show that neurons with sex steroid receptors are the most abundantly distributed in POA among brain regions in the medaka brain. Therefore, POA is suggested to play the most important role in the feedback regulation of the HPG axis.

Conclusions

In the present Chapter, the distribution of *erα, erβ1, erβ2*, *arα* and *arβ* mRNA expressing neurons in female medaka brain was examined. In the ER and/or AR expressing neurons, the gene transcription and/or possibly firing activities may be regulated by estrogen or androgen, and these neurons are suggested to function as regulators of various neural functions, especially reproductive functions, according to the brain regions (BO for regulating motivation for some sexual behaviors; Vv, Vd, Vs, Vp for regulating motor aspects of various sexual behaviors; POA for regulating the HPG axis). In addition to reproduction, it has been reported that both ER and AR are involved in many other functions such as aggression, or feeding. However, further studies are necessary to understand the regulatory mechanisms of these neural functions

by sex steroid hormones. For example, the identification of the neurotransmitters and precise analysis of the neural networks formed by the ER and/or AR expressing neurons will provide important information. The results of the present Chapter provide important bases for those future studies that should provide essential information toward the understanding of the regulatory mechanisms of various neural functions that are modulated or even initiated by estrogen or androgen.

Figure legends

Fig. 1

An atlas of medaka brain based on the Nissl stained frontal sections of the female medaka. Frontal sections at different levels are arranged rostrocaudally from (A) to (J). The levels of the sections are indicated in the top inset (lateral view of the brain). The boxed areas with alphabets in A-I correspond to the panels in Fig. 2 (A–H).

Fig. 2

Nissl stained sections showing regions where the labeled neurons were mainly distributed in the present Chapter (A-H). The areas correspond to the panels (A–H) are boxed with alphabets in Fig. 1 (A-I). Scale bar, A-C 100 μm, D-E 50 μm, F 200 μm, G-H 100 μm.

Fig. 3

Schematic illustrations and photomicrographs showing the absence of *erα* or **arα** mRNA-expressing neurons (ERα or ARα neurons) in nucleus of solitary tract in the hindbrain and spinal cord of female medaka, demonstrated by *in situ* hybridization. Frontal sections at different levels are arranged rostrocaudally from (A) to (C). The levels of the sections are indicated in the top inset (lateral view of the brain). The photomicrographs show representative frontal sections correspond to the illustrations on the right. The areas corresponding to the photographs are boxed in illustrations.
I performed the same analyses for all other subtypes of *er* and *ar*, but I did not find any positive signals for these receptors (data not shown). Scale bar, 100 μm.

Fig. 4

Schematic illustrations showing the distribution of *erα* mRNA-expressing neurons (ERα neurons) in the female medaka brain, demonstrated by *in situ* hybridization. For each representative frontal section of the brain, ER and/or AR neurons are indicated by dots, squares, or triangles, but they do not reflect the actual numbers of neurons.
Frontal sections at different levels are arranged rostrocaudally from (A) to (J). The levels of sections are indicated in the top inset (lateral view of the brain). The areas boxed with alphabets in (A-J) correspond to the panels in Fig. 5 (A–F).
Most rostrally, the distribution pattern and cell size of ERα neurons (A) were similar to the terminal nerve (TN)-GnRH neurons. The ERα neurons were distributed in the ventral telencephalon, from Vd to Vp (B-D). The *erα* mRNA was expressed in the rostral part of POA (rPOp) at its highest level in the brain (C). In the diencephalon and mesencephalon, the ERα neurons were observed in the NVT, NPPv (F-G), and the area dorsal to flm (H). In addition, *erα* mRNA was expressed along the ver (I-J) and in RFm (J) in the hindbrain.

Fig. 5

(A-F) Photomicrographs showing the distribution of *erα* mRNA-expressing neurons (ERα neurons) in the female medaka brain, demonstrated by *in situ* hybridization. The areas correspond to the panels (A–F) are boxed with alphabets in Fig. 4 (A-J). (G-I) Schematic illustrations and pictures showing ERα neurons that were expressed along the ver in more detail. (G) Frontal section of the hindbrain. Regions correspond to panels (H) and (I) are boxed in Fig. 4. Higher magnification slides of Nissl stained section (H)

and ERα-expressing cells (I, arrow heads) in the region that is close to ver suggest that most of these ERα positive small cells are neurons. Scale bar, A-F 50 μm, H-I 25 μm.

Fig. 6

Double-label *in situ* hybridization analysis showing co-localization of *gnrh3* and *erα* mRNA in TN-GnRH neurons. (A) Demonstration of *gnrh3* mRNA expression in the TN-GnRH neurons. The positive signal was visualized with Alexa Fluor488 (green). (B) Demonstration of *erα* mRNA expression on the same section. The expression signal was visualized with Fast-Red (magenta). (C) The merged image of (A) and (B), showing the co-localization (white) of *gnrh3* (green) and *erα* (magenta). Panels (D) and (E) show control slides for the specificity of the antisense probe for *erα* mRNA, antisense probe for *gnrh3* (D) and with sense probe for *erα* (E), and merged image (F). Panels (G) and (H) demonstrate the specificity of antisense probe for *gnrh3* mRNA. These panels show the area (rostral part of POp) where only *erα*, but not *gnrh3*, mRNA were labeled in another section of the same slide as shown in (A-C). (G): antisense probe for *gnrh3*, (H): antisense probe for *erα* (I): merged image. Scale bar, 20 μm.

Fig. 7

Schematic illustrations showing the distribution of *erβ1* mRNA-expressing neurons (ERβ1 neurons) in the female medaka brain, demonstrated by *in situ* hybridization. Frontal sections at different levels are arranged rostrocaudally from (A) to (H). The levels of the sections are indicated at the top inset (lateral view of the brain). The areas boxed with alphabets correspond to the panels in Fig. 8 (A–F). The ERβ1 neurons were distributed in the ventral telencephalon, from Vv (A) and Vd to Vp (A-C). The *erβ1*

mRNA was expressed in the rostral part of POA (rPOp) at its highest level in the brain (B). In hypothalamus, the ERβ1 neurons were observed in the NVT and NPPv (F-F). In addition, *erβ1* mRNA was expressed along the ver (G-H).

Fig. 8

Photomicrographs showing the distribution of *erβ1* mRNA-expressing neurons (ERβ1 neurons) in the female medaka brain, demonstrated by *in situ* hybridization.
The areas correspond to the panels (A–F) are boxed with alphabets in Fig. 7 (A-H).
Scale bar, 50 μm.

Fig. 9

Schematic illustrations showing the distribution of *erβ2* mRNA-expressing neurons (ERβ2 neurons) in the female medaka brain, demonstrated by *in situ* hybridization. Frontal sections at different levels are arranged rostrocaudally from (A) to (H). The levels of the sections are indicated at the top inset (lateral view of the brain). The areas boxed with alphabets correspond to the panels Fig. 10 (A–F). The ERβ2 neurons were distributed in the ventral telencephalon, from Vd to Vp (A-C). The *erβ2* mRNA was expressed in the rostral part of POA (rPOp) at its highest level in the brain (B). In the diencephalon, the ERβ2 neurons were observed in the VM (E), NVT, and NPPv (E-F). In addition, *erβ2* mRNA was expressed along the ver (G-H) in the hindbrain.

Fig. 10

Photomicrographs showing the distribution of *erβ2* mRNA-expressing neurons (ERβ2 neurons) in the female medaka brain, demonstrated by *in situ* hybridization.

The areas correspond to the panels (A–F) are boxed with alphabets in Fig. 9 (A-H). Scale bar, 50 μm.

Fig. 11

Schematic illustrations showing the distribution of *arα* mRNA-expressing neurons (ARα neurons) in the female medaka brain, demonstrated by *in situ* hybridization. Frontal sections at different levels are arranged rostrocaudally from (A) to (J). The levels of sections are indicated in the top inset (lateral view of the brain). The areas boxed with alphabets correspond to the panels Fig. 12 (A–H). Most rostrally, ARα neurons were localized in Dl and the granule cell layer of BO (A). The ARα neurons were distributed in the ventral telencephalon, from Vv (B) and Vd to Vp (B-D). The *arα* mRNA was expressed in the rostral part of POA (rPOp) at its highest level in the brain (C). ARα neurons were also observed within EmT (E). In the diencephalon and mesencephalon, the ARα neurons were observed in DM (F), NVT, NPPv, TO (stratum periventriculare) (F-G), TS (G) and dorsal to flm (H). In addition, *arα* mRNA was expressed along the ver (I-J) and in RFm (J) in the hindbrain.

Fig. 12

Photomicrographs showing the distribution of *arα* mRNA-expressing neurons (ARα neurons) in the female medaka brain, demonstrated by *in situ* hybridization.
The areas correspond to the panels (A–H) are boxed with alphabets in Fig. 11 (A-J). Scale bar, 50 μm.

Fig. 13

(A) Nissl stained section showing torus semicircularis (TS) in female medaka. (B) The broken lines in (A) show the nuclear boundary of TS, indicating that TS of medaka consists of three different subdivisions (outlined by broken lines). (C) and (D) show the distributions of *arα* mRNA-expressing neurons (ARα neurons) in the female medaka brain, demonstrated by *in situ* hybridization. (C) shows rostral part, and (D) shows caudal part of TS. ARα neurons were localized in the lateral region of all three subdivisions of the TS. Scale bar, 50 μm.

Fig. 14

Schematic illustrations showing the distribution of *arβ* mRNA-expressing neurons (ARβ neurons) in the female medaka brain, demonstrated by *in situ* hybridization. Frontal sections at different levels are arranged rostrocaudally from (A) to (H). The levels of the sections are indicated at the top inset (lateral view of the brain). The areas boxed with alphabets correspond to the panels Fig. 15 (A–E). The ARβ neurons were distributed in the ventral telencephalon, from Vv(A) and Vd to Vp (A-C). The ARβ mRNA was expressed in the rostral part of POA (rPOp) at its highest level in the brain (B). ARβ neurons were also observed within EmT (D). In the diencephalon, the ARβ neurons were observed in the DM (E), TO (stratum periventriculare), NVT, and NPPv (E-F). In addition, *arβ* mRNA was expressed along the ver (H) and in RFm (H) in the hindbrain.

Fig. 15

Photomicrographs showing the distribution of *arβ* mRNA-expressing neurons (ARβ neurons) in the female medaka brain, demonstrated by *in situ* hybridization.

The areas correspond to the panels (A–E) are boxed with alphabets in Fig. 14 (A-H).

Scale bar, 50 μm.

Abbreviations

AP	area postrema
BO	olfactory bulb
ca	anterior commissure
cc	central canal
CE	corpus of the cerebellum
dDm	dorsal region of the medial part of dorsal telencephalic area
dDl	dorsal region of Dl
Dl	lateral part of dorsal telencephalic area
DM	dorsomedial thalamic nucleus
Dp	posterior part of dorsal telencephalic area
E	epiphysis
EmT	eminentia thalami
fd	dorsal funiculus
flm	medial longitudinal fasciculus
GR	corpus glomerulosus
NAT	anterior tuberal nucleus
NCC	commissural nucleus of Cajal
NDTL	diffuse nucleus of torus lateralis
nII	optic nerve
NIXm	nucleus of hypoglossal nerve
NXm	motor nucleus of vagal nerve
NPPv	periventricular posterior nucleus
NRL	nucleus of lateral recess
NVT	ventral tuberal nucleus
POA	preoptic area
POm	magnocellular preoptic nucleus
POp	parvocellular preoptic nucleus
RFm	medial reticular zone
rPOp	rostral part of POp
SPV	periventriculare stratum
TN	terminal nerve
TO	optic tectum
TS	torus semicircularis
TSc	central nucleus of TS
TSe	external nucleus of TS
TSvl	ventrolateral nucleus of TS
V	ventral telencephalic area
Vd	dorsal part of ventral telencephalic area
ver	rhombencephalic ventricle
VIIL	facial lobes
VM	ventromedial thalamic nucleus
Vp	postcommissural part of V
Vs	supracommissural part of V
Vv	ventral part of V
XL	vagal lobes

Table 1

Primer information for *in situ* hybridization probe template

Gene	Forward primer	Reverse primer
erα	5’-AGTCCCTCTTTATGACCTCC	5’-AAATAGAAACAGGAATTGCC
erβ1	5’-CATGGTGCCTTTGTACGACCTGC	5’-GTTTACTCATGACTGGAGCGGCCA
erβ2	5’-TGGACGCCAACACGCCCAGCA	5’-GGCGTGTTTTACTGCGGATTTGAC
arα	5’-GGTTTAGGAGGAGTTTGCGGCTCT	5’-TTGGAATAGGGTGTCCTGGGCAT
arβ	5’-AGCTTGGCGGAAAAACAAGAAACG	5’-CACACAGGTGGTTCTGCTTACCT

Table 2

Distribution of estrogen receptors (ERs) and Androgen Receptors (ARs)

in the Brain of female medaka

Brain area	ERα	ERβ1	ERβ2	ARα	ARβ
Dl	-	-	-	+	-
TN	+	-	-	-	-
BO	-	-	-	+	-
Vd	+	+	+	+	+
Vs	+	+	+	+	+
Vp	+	+	+	+	+
Vv	-	+	-	+	+
rPOp	+	+	+	+	+
POm	+	+	+	+	+
EmT	-	-	-	+	+
TO (SPV)	-	-	-	+	+
TS	-	-	-	+	-
DM	-	-	-	+	+
VM	-	-	+	-	-
NPPv	+	+	+	+	+
NVT	+	+	+	+	+
area dorsal to flm	+	-	-	+	-
area close to ver	+	+	+	+	+
RFm	+	-	-	+	+

+, present; -, absent

Fig.1

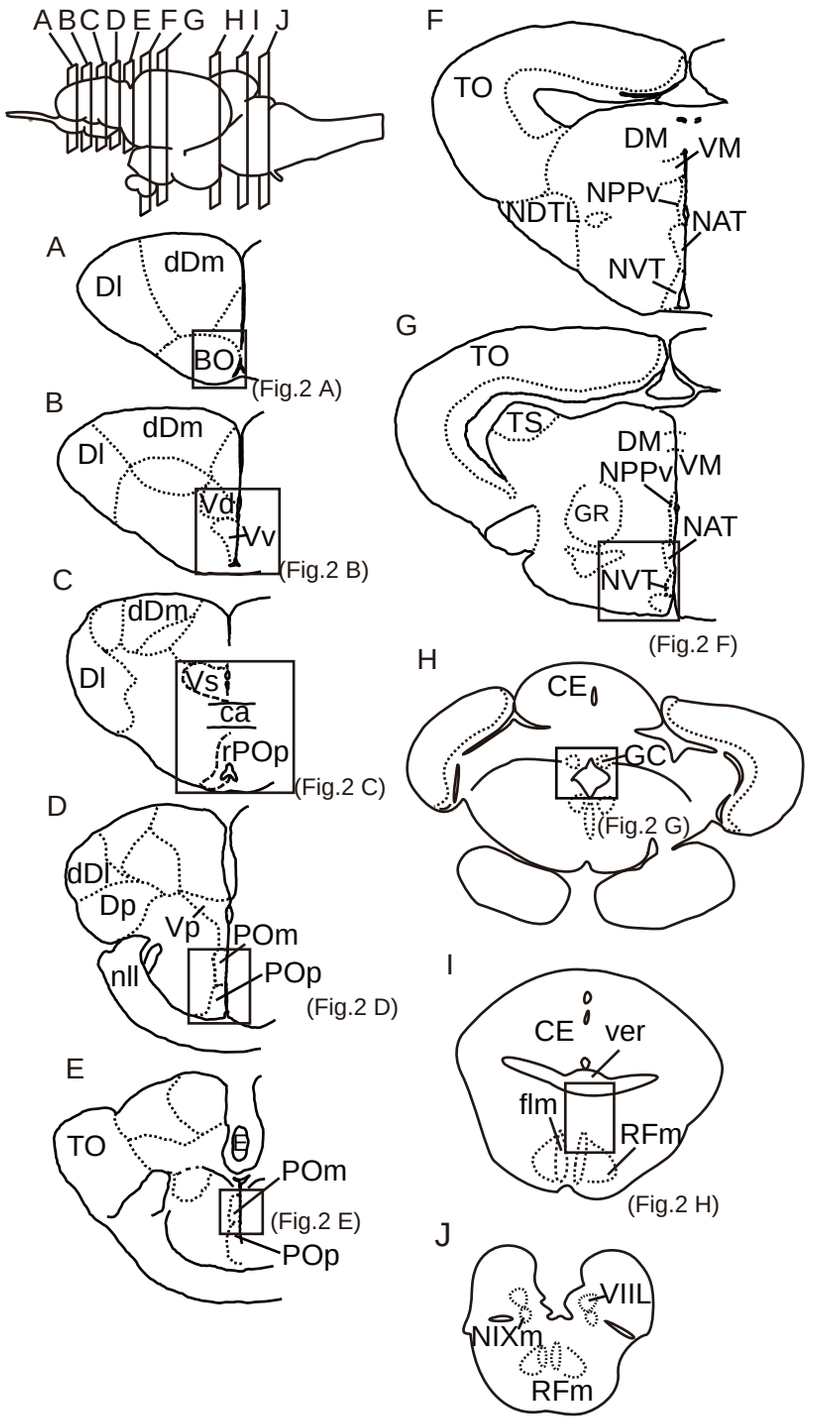

A B C D E F G H I J
A
dDm
Dl
BO
(Fig.2 A)
B
dDm
Dl
Vd
Vv
(Fig.2 B)
C
dDm
Dl
Vs
ca
rPOp
(Fig.2 C)
D
dDl
Dp
Vp
POm
nll
POp
(Fig.2 D)
E
TO
POm
(Fig.2 E)
POp
F
TO
DM
VM
NPPv
NDTL
NAT
NVT
G
TO
TS
DM
VM
NPPv
GR
NAT
NVT
(Fig.2 F)
H
CE
GC
(Fig.2 G)
I
CE
ver
flm
RFm
(Fig.2 H)
J
VIIL
NIXm
RFm

Fig.2

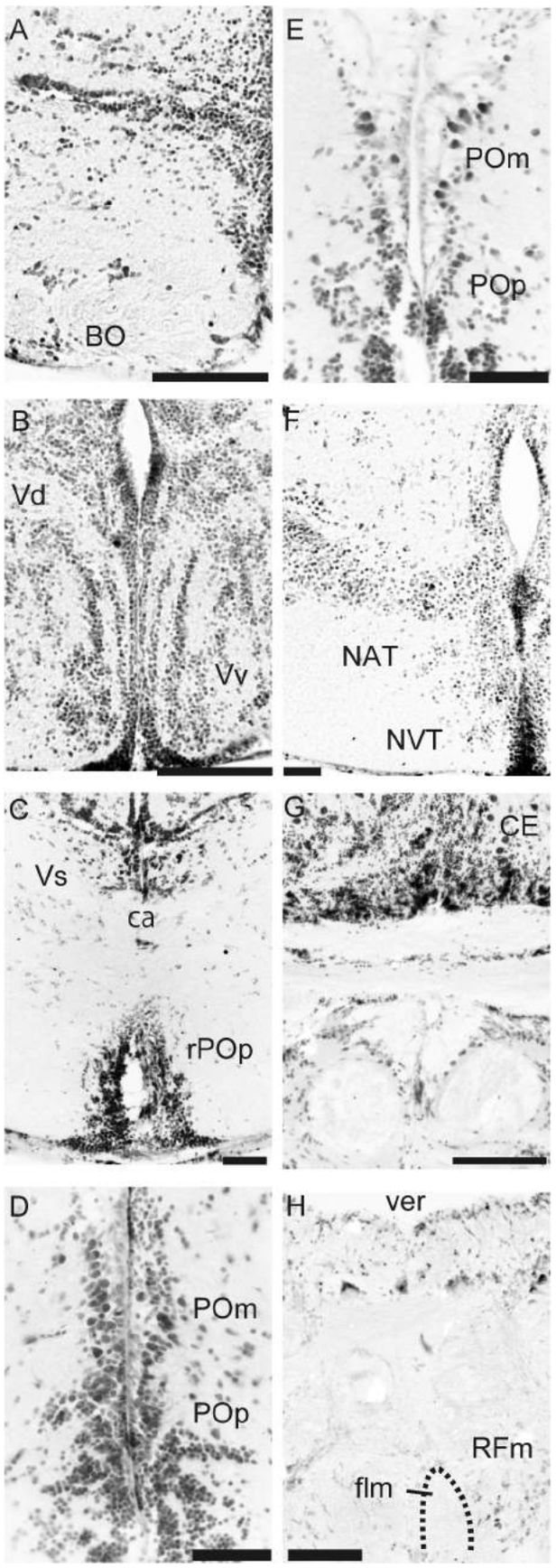

A
BO
E
POm
POp
B
Vd
Vv
F
NAT
NVT
C
Vs
ca
rPOp
G
CE
D
POm
POp
H
ver
RFm
flm

Fig.3

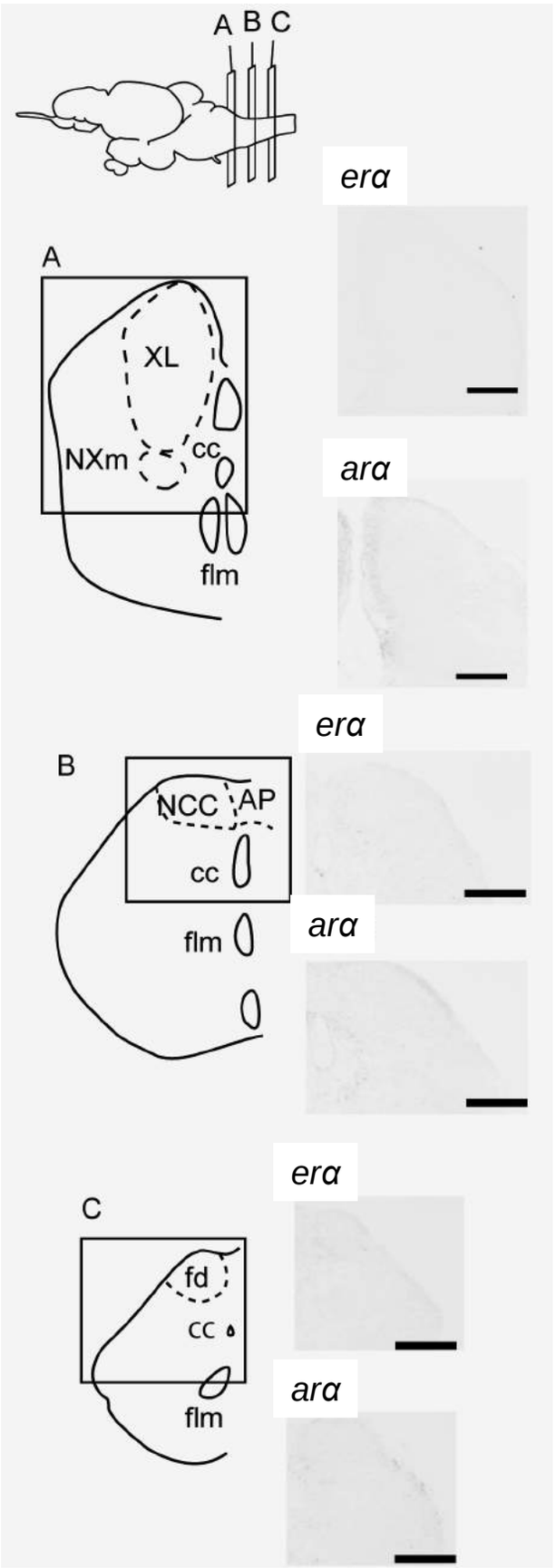

A B C
erα
A
XL
NXm
cc
flm
arα
erα
B
NCC
AP
cc
flm
arα
erα
C
fd
cc
flm
arα

Fig.4

erα

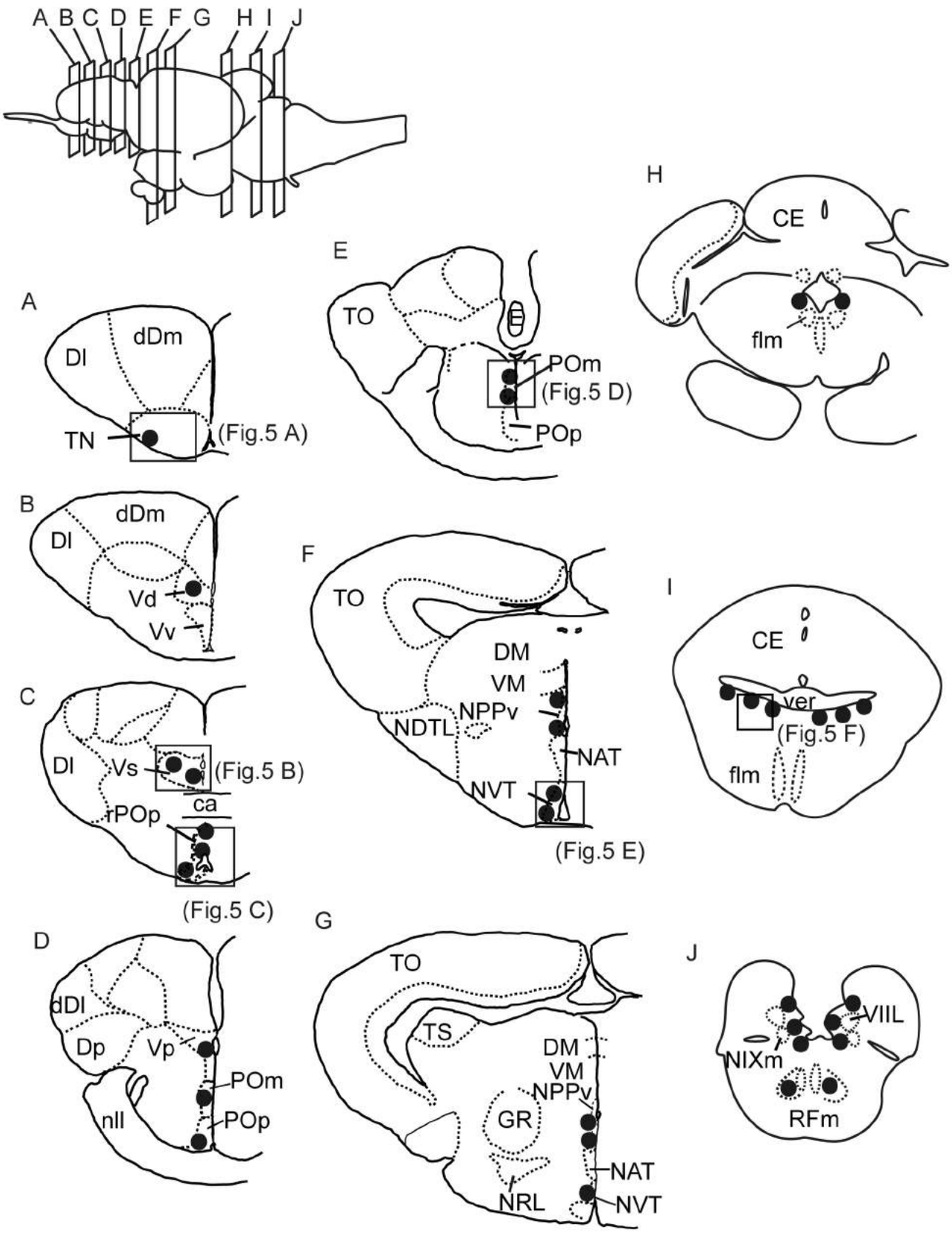

A B C D E F G H I J
A
dDm
Dl
TN
(Fig.5 A)
B
dDm
Dl
Vd
Vv
C
Dl
Vs
(Fig.5 B)
ca
rPOp
(Fig.5 C)
D
dDl
Dp
Vp
POm
nll
POp
E
TO
POm
(Fig.5 D)
POp
F
TO
DM
VM
NPPv
NDTL
NAT
NVT
(Fig.5 E)
G
TO
TS
DM
VM
NPPv
GR
NAT
NRL
NVT
H
CE
flm
I
CE
ver
(Fig.5 F)
flm
J
VIIL
NIXm
RFm

Fig.5

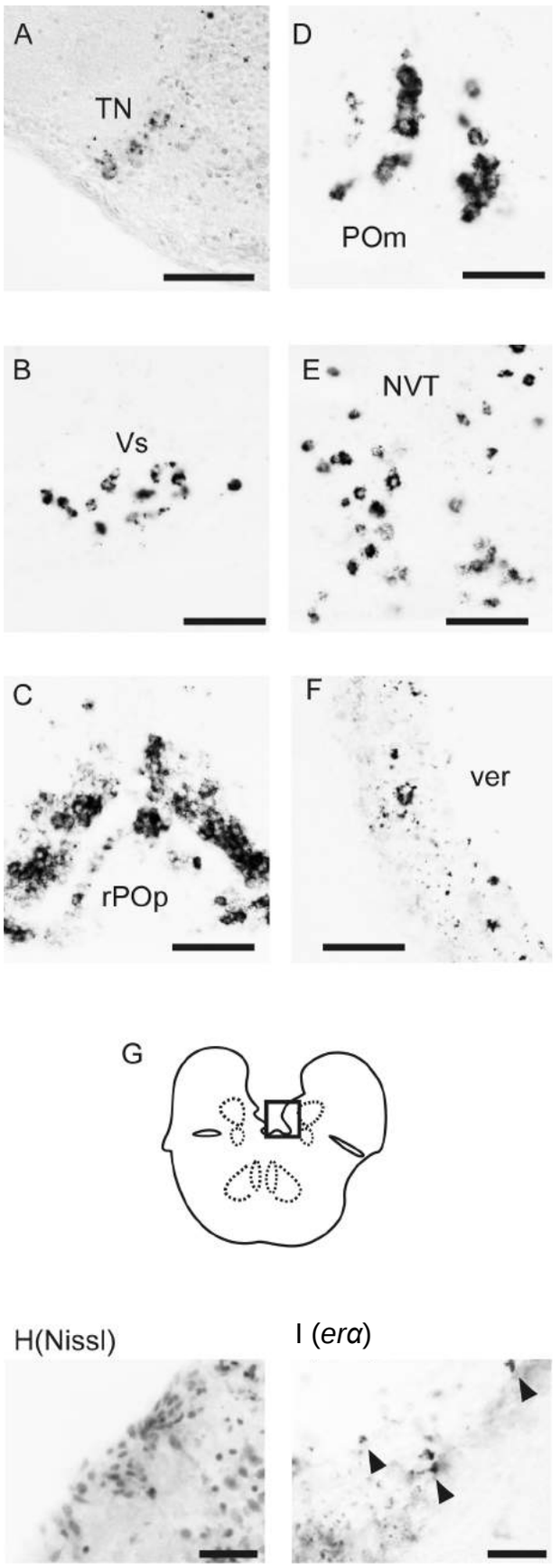


Fig.6

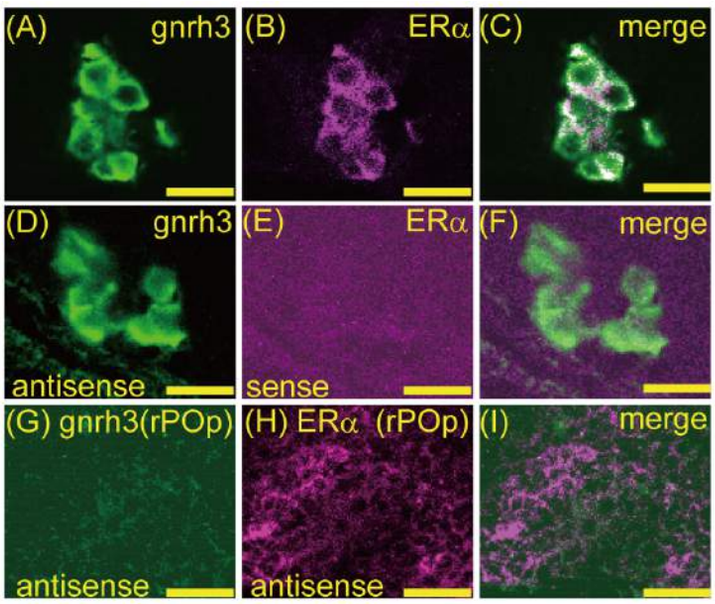

(A) gnrh3
(B) ERα
(C) merge
(D) gnrh3
antisense
(E) ERα
sense
(F) merge
(G) gnrh3(rPOp)
antisense
(H) ERα (rPOp)
antisense
(I) merge

Fig.7

erβ1

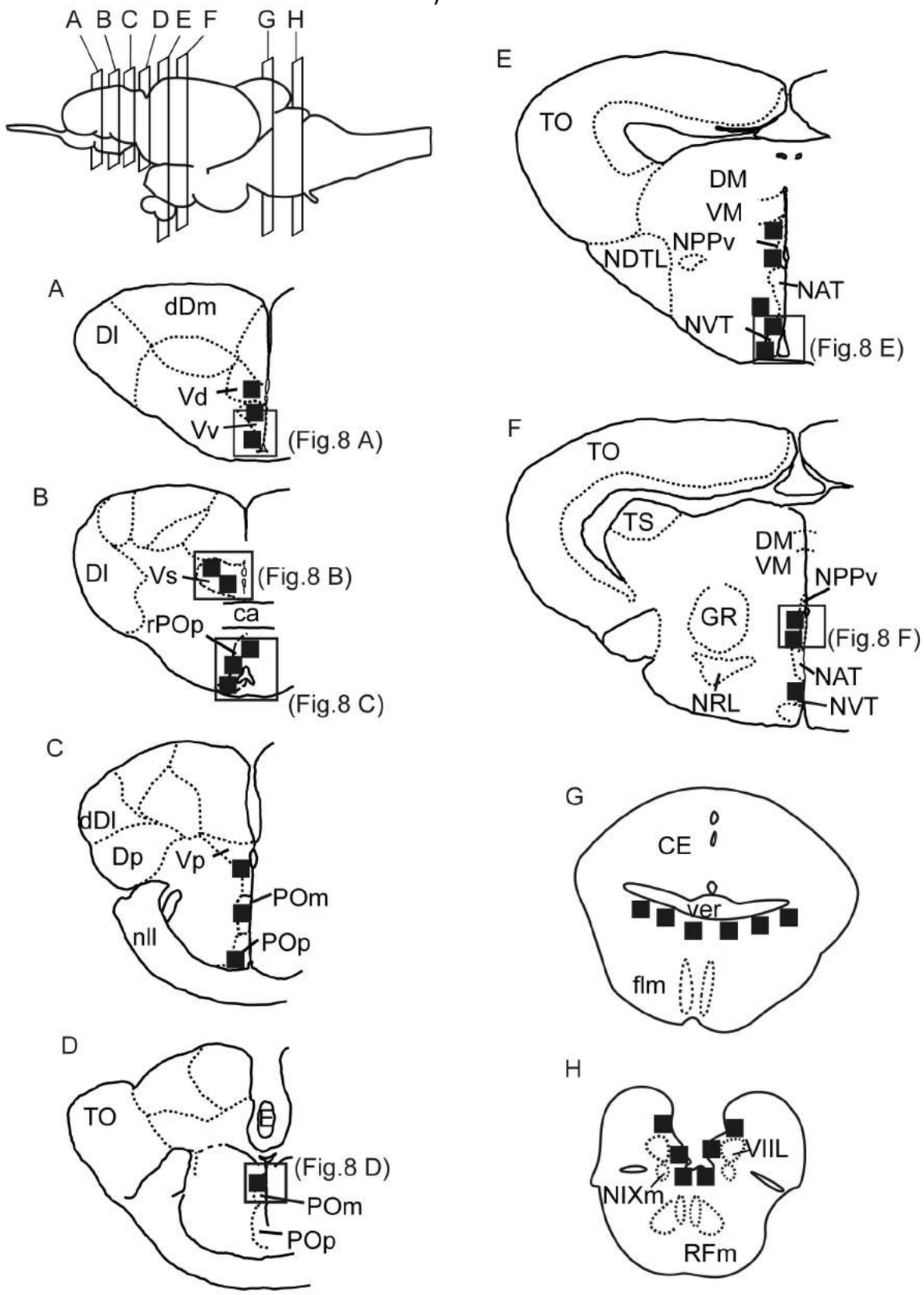

A B C D E F G H
A
dDm
Dl
Vd
Vv
(Fig.8 A)
B
Dl
Vs
(Fig.8 B)
ca
rPOp
(Fig.8 C)
C
dDl
Dp
Vp
POm
nll
POp
D
TO
(Fig.8 D)
POm
POp
E
TO
DM
VM
NPPv
NDTL
NAT
NVT
(Fig.8 E)
F
TO
TS
DM
VM
NPPv
GR
(Fig.8 F)
NAT
NRL
NVT
G
CE
ver
flm
H
VIIL
NIXm
RFm

Fig.8

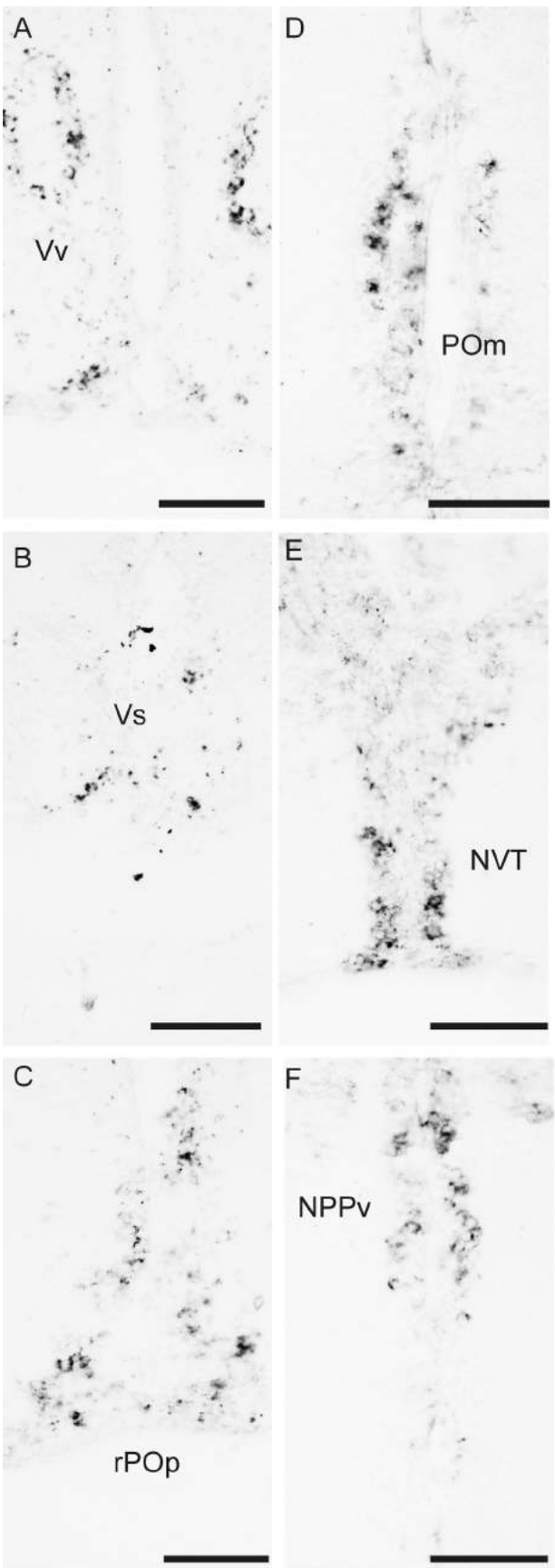
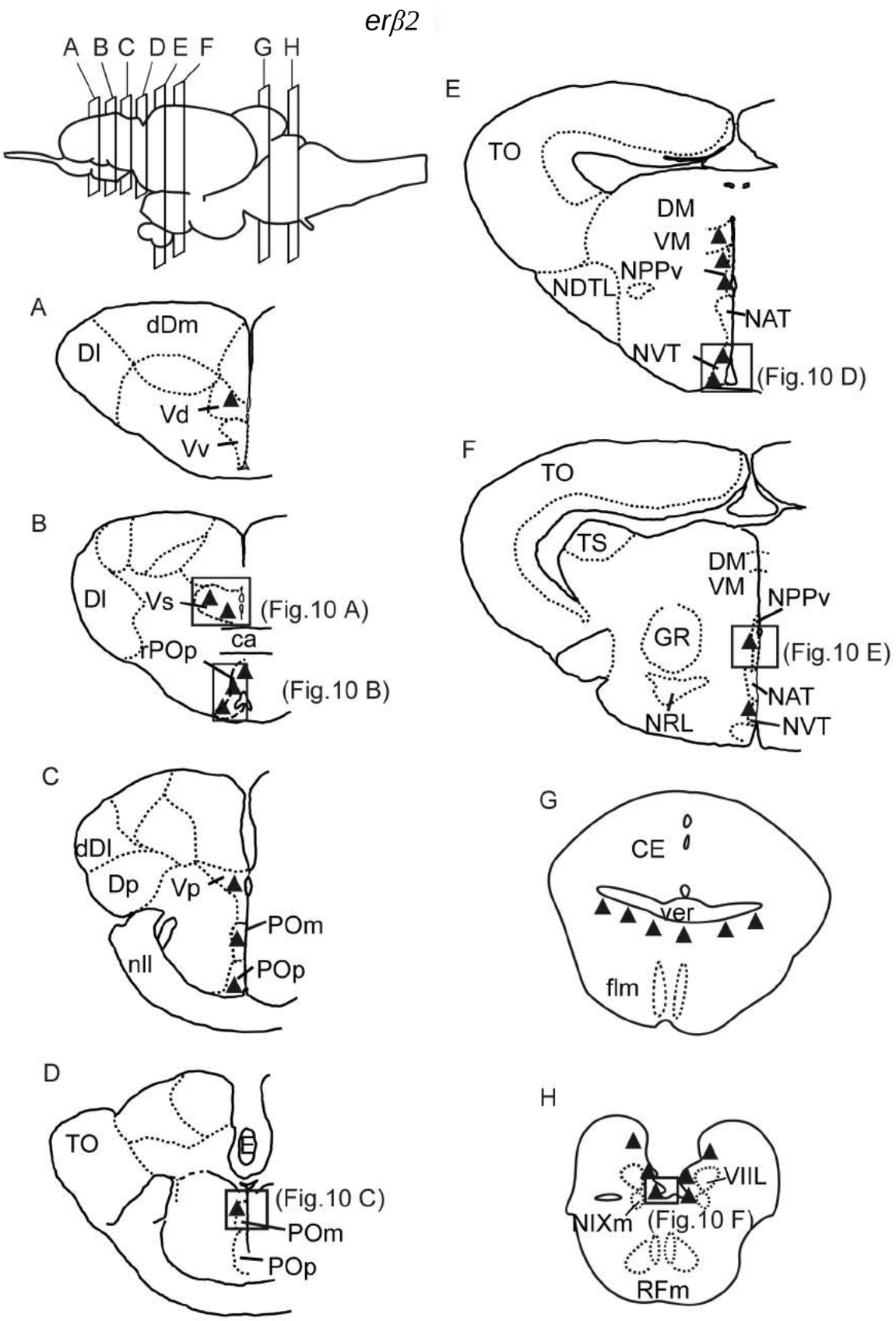


Fig.9


erβ2
A B C D E F G H
A
dDm
Dl
Vd
Vv
B
Dl
Vs
(Fig.10 A)
ca
rPOp
(Fig.10 B)
C
dDl
Dp
Vp
POm
nll
POp
D
TO
(Fig.10 C)
POm
POp
E
TO
DM
VM
NPPv
NDTL
NAT
NVT
(Fig.10 D)
F
TO
TS
DM
VM
NPPv
GR
(Fig.10 E)
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NRL
NVT
G
CE
ver
flm
H
VIIL
NIXm
(Fig.10 F)
RFm

Fig.10

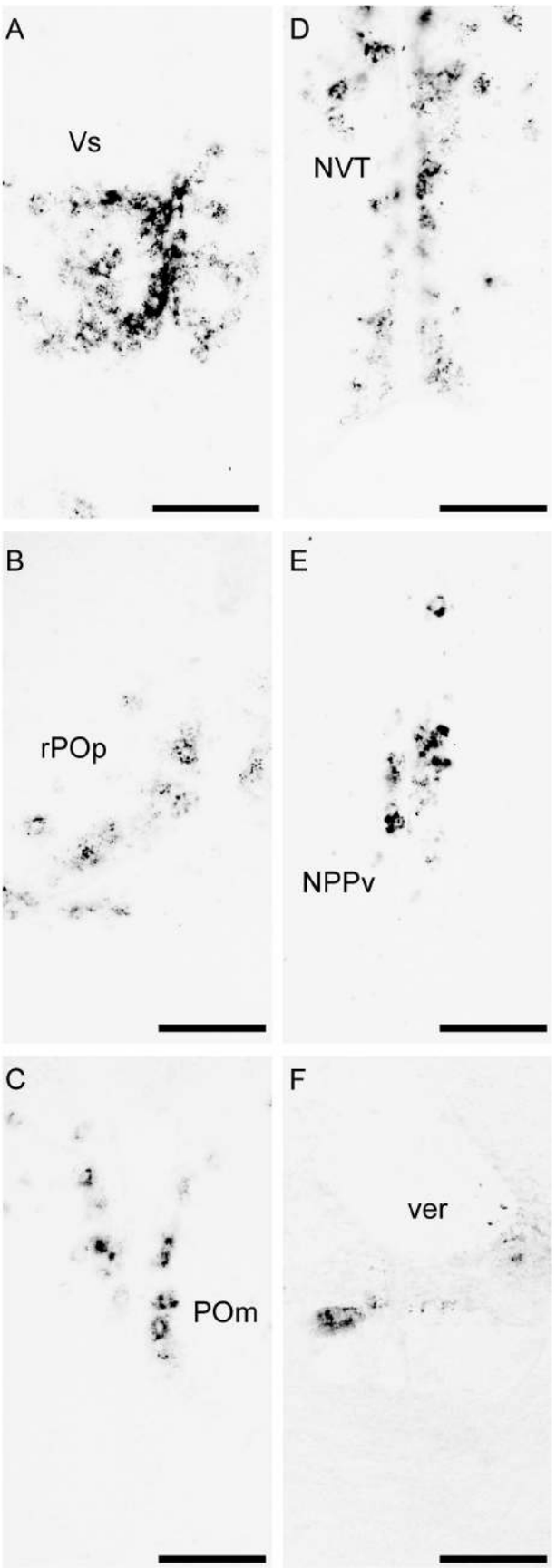
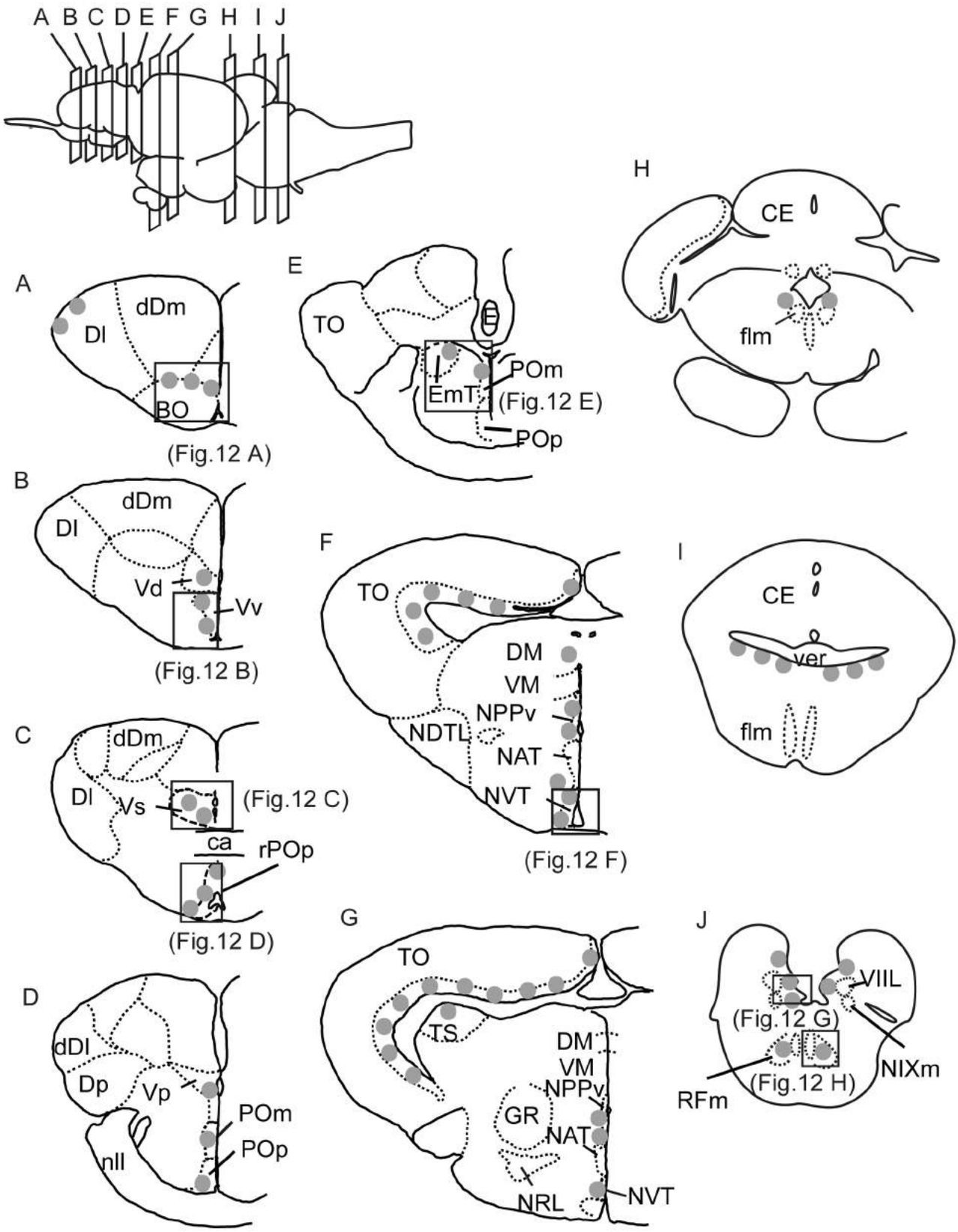


Fig.11

arα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dDm
Dl
BO
(Fig.12 A)
B
dDm
Dl
Vd
Vv
(Fig.12 B)
C
dDm
Dl
Vs
(Fig.12 C)
ca
rPOp
(Fig.12 D)
D
dDl
Dp
Vp
POm
POp
nll
E
TO
POm
EmT
(Fig.12 E)
POp
F
TO
DM
VM
NPPv
NDTL
NAT
NVT
(Fig.12 F)
G
TO
TS
DM
VM
NPPv
GR
NAT
NRL
NVT
H
CE
flm
I
CE
ver
flm
J
VIIL
(Fig.12 G)
(Fig.12 H)
NIXm
RFm

Fig.12

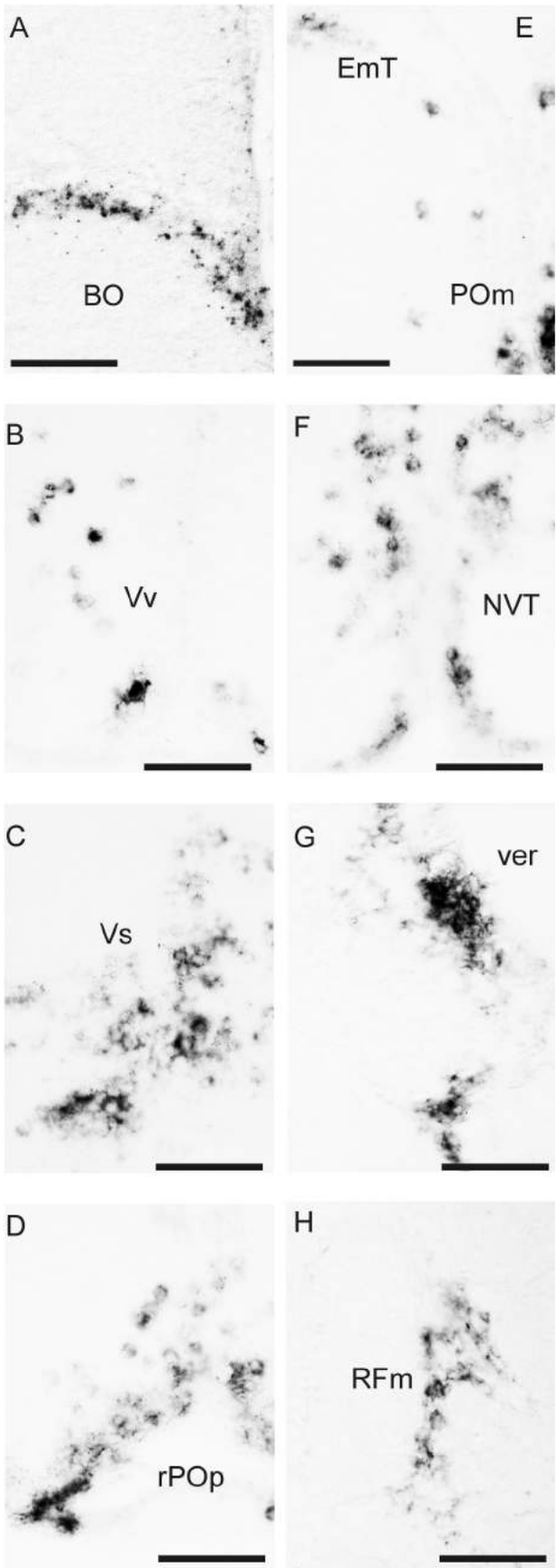


Fig.13

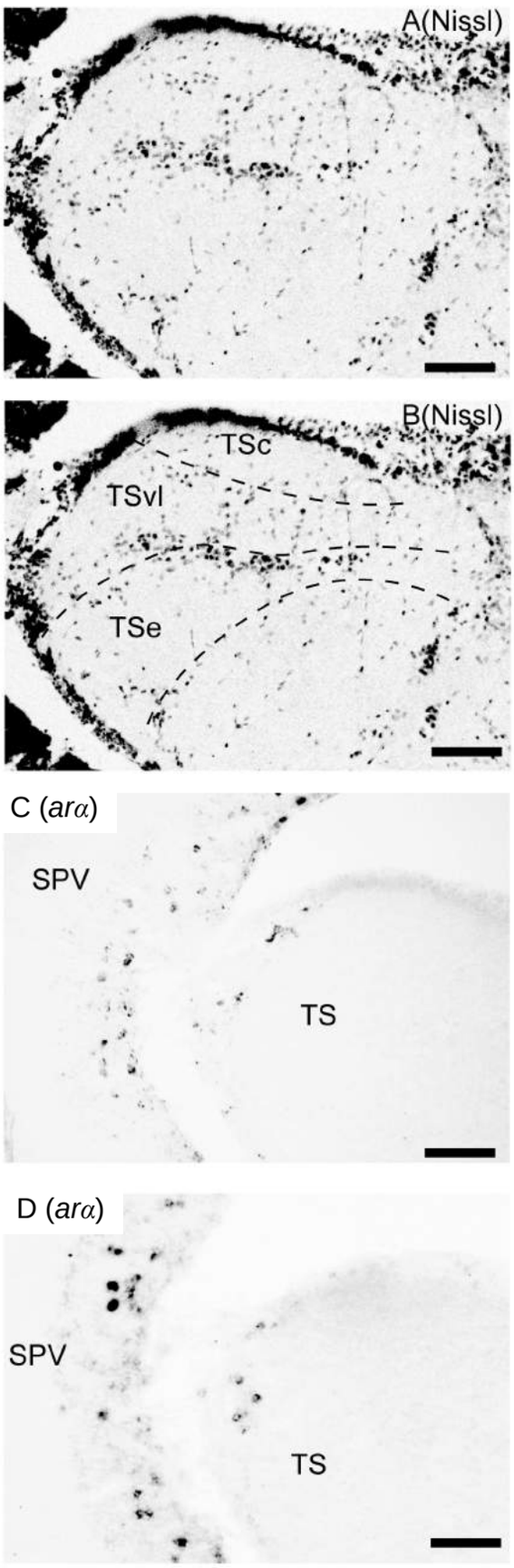
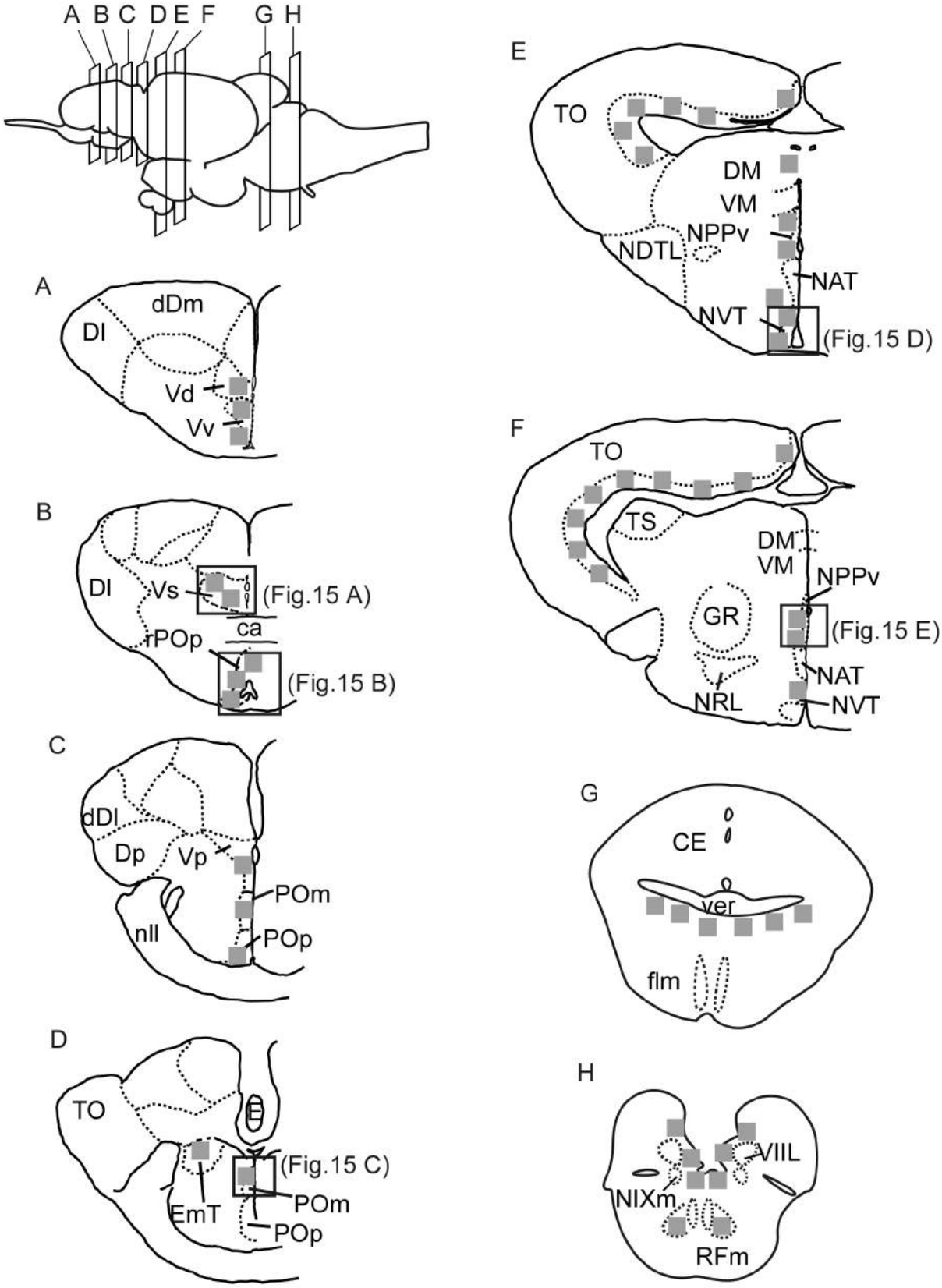


Fig.14

arβ


A B C D E F G H
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dDm
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Vd
Vv
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Dl
Vs
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ca
rPOp
(Fig.15 B)
C
dDl
Dp
Vp
POm
nll
POp
D
TO
(Fig.15 C)
EmT
POm
POp
E
TO
DM
VM
NPPv
NDTL
NAT
NVT
(Fig.15 D)
F
TO
TS
DM
VM
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NAT
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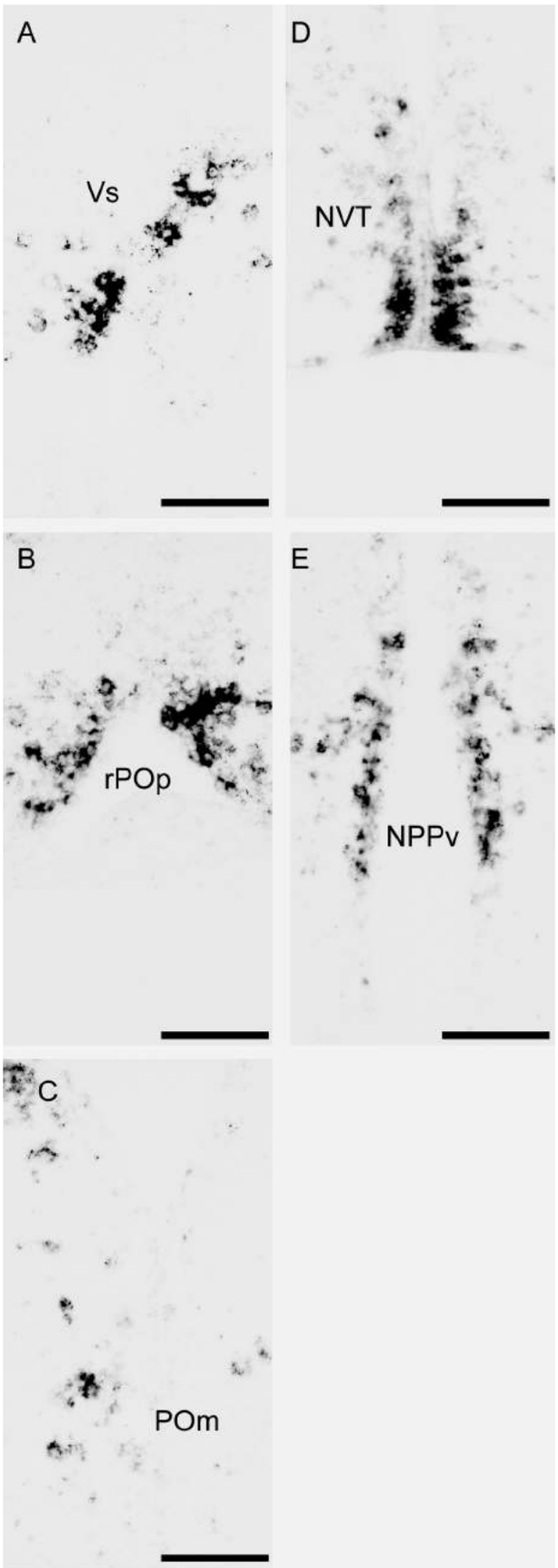
G
CE
ver
flm
H
VIIL
NIXm
RFm

Fig.15



Chapter 2

Estrogen directly acts on the gonadotropes in the medaka pituitary

Abstract

There are two type of gonadotropins from the pituitary, follicle stimulating hormone (FSH) and luteinizing hormone (LH), and it has been suggested that FSH stimulates gonads and induces maturation of ovarian follicles, and LH triggers ovulation. These two gonadotropins are therefore considered to act on the gonads at different stages of gonadal maturation. Then, LH and FSH are considered to be regulated by different mechanisms. In addition to well-known regulations by hypophysiotropic neurons such as gonadotropin releasing hormone (GnRH1) neurons, LH and FSH cells are also suggested to be regulated by direct feedback effects of gonadal sex steroid hormone, estrogen. Thus, elucidation of both direct and indirect (via GnRH1 neurons: HPG axis regulation) regulations by sex steroids are important for further understanding of the steroid feedback systems in the regulatory mechanisms of reproductions. However, compared to the indirect regulation via the HPG axis, details of the direct actions of estrogen on the pituitary gonadotropes remain to be clarified. In the present Chapter, I analyzed the effects of estrogen on the LH cells and FSH cells, using medaka. By taking advantage of small pituitary of medaka, I used a whole pituitary preparation which maintains intercellular communications within the pituitary but is separated from the hypothalamic regulations. First, I demonstrated by *in situ* hybridization analysis that both LH cells and FSH cells express estrogen receptor α (ERα). Second, I carried out estrogen treatment in the intact whole pituitary preparations and demonstrated that estrogen directly inhibits the transcription of *fshβ* mRNA, but not that of *lhβ* mRNA. Therefore, it is suggested that the transcription of *lhβ* is mainly upregulated via the HPG axis, while that of *fshβ* is regulated more strongly by the direct action of gonadal estrogen. By regulations from these different systems, FSH and LH are released at

different timings from each other and stimulate gonads to induce maturation of ovarian follicles or ovulation, respectively.

Introduction

Gonadal maturation is strictly regulated by gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), released from the pituitary. LH and FSH are known to exhibit different actions on gonads. In female, FSH induces maturation of oocytes, whereas LH mainly causes the follicular rupture or ovulation (Baird, 1983). These functions of gonadotropins are suggested to be conserved in jawed vertebrates despite different reproductive strategies among species. In teleosts, two distinct types of gonadotropins were first identified in late 1980's (Suzuki et al., 1988). Studies in teleosts have so far suggested that FSH induces growth of follicles, whereas LH induces final oocyte maturation and ovulation, as in mammals (Prat et al., 1996; Breton et al., 1998; Yaron et al., 2003). In addition, the serum levels of LH and FSH have been suggested to change characteristically and with different timings in accordance with the gonadal status in both mammals and teleosts (Murr et al., 1973; Baird, 1983; Hansel and Convey, 1983; Golan et al., 2014; Stachenfeld and Taylor, 2014).

In female vertebrates, the information of gonadal status is transmitted by the circulating sex steroid hormones, especially estrogen. Gonadal estrogen has been suggested to stimulates hypothalamic neurons via estrogen receptors in the brain and regulate the release of hypophysiotropic gonadotropin releasing hormone (called as GnRH1 in most animals) from the GnRH1 neurons to stimulate the releasing of LH/FSH. This regulatory pathway has long been known as the hypothalamo-pituitary-gonadal (HPG) axis. In addition to this type of indirect estrogenic action, there has been some reports in mammals and teleosts that estrogen also controls expressions of LH/FSH by directly acting on the gonadotropes (Miller et al., 1983; Nett et al., 2002). Previous knockout studies clearly showed that both of these pathways, indirect and

direct pathways, are important for the regulation of reproduction. It has been reported that neuron-specific knockout of estrogen receptor (ER) α causes infertility in mice (Wintermantel et al., 2006). On the other hand, gonadotrope-specific knockout of ERα also causes infertility or subfertility (Gieske et al., 2008; Singh et al., 2009). Therefore, cooperative actions of both indirect and direct pathways of gonadal estrogenic activation are considered to be important for the regulations of reproduction.

However, feedback effects of estrogen on gonadotropins are not common to mammals and teleosts. Studies in mammals using gonadectomy have suggested that estrogen generally has negative feedback effects on both LH and FSH (Shupnik, 1996). On the other hand, in teleosts, gene expression and plasma concentrations of LH are generally up-regulated by estrogen, whereas FSH shows various responses depending on species or reproductive stages (Yaron et al., 2003). Such different responses of gonadotropins to estrogen may be caused by differences in stimulations from hypothalamus and/or by the presence of direct actions of estrogen. For the analysis of direct estrogenic actions on pituitary, previous studies mainly used sex steroid treatment in dispersed pituitary cell culture (Miller et al., 1983; Lin and Ge, 2009). However, these dispersed cultured cells are considered to have lost autocrine/paracrine interactions among the pituitary cells that have been suggested to be involved in the regulation of gonadotropes (Bilezikjian et al., 2004; Yuen and Ge, 2004; Lin and Ge, 2009). Thus, studies of dispersed pituitary cells may not be appropriate for analyzing functions of sex steroids in the pituitary precisely. To solve these problems, I here chose medaka as a model animal. Medaka have the following unique characteristics, which provide ideal approaches for the analysis of regulation of gonadotropes. The medaka pituitaries, even in adults, are small enough to be incubated as an intact whole pituitary

preparation for a couple of hours without dispersing or slicing. A method of analysis in whole pituitary preparation has already been established, in which expression levels of LH, FSH mRNA can be quantitatively analyzed after drug treatment (Karigo et al., 2012). In addition, the regulatory systems for reproductive functions of medaka have been well documented; effects of hypophysiotropic GnRH1 on gonadotropes have been already analyzed in detail (Karigo et al., 2012; Karigo et al., 2014).

In the present Chapter, I focused on the direct effects of sex steroids on the gonadotropes. First, I analyzed the expression of the ERα in the gonadotropes in medaka. Then, I analyzed direct effects of estrogen on the expression of *lhβ* mRNA and *fshβ* mRNA by using intact whole pituitary preparation.

.

Materials and methods

Animals

Female wild type d-rR medaka (*Oryzias latipes*) were maintained under a 14 h light and 10 h dark photoperiod at a water temperature of 27°C. Fish were fed twice or three times a day with live brine shrimp and flake food. All animals were maintained and used in accordance with the guidelines of the University of Tokyo for the Use and Care of Experimental Animals.

***In situ* hybridization analysis**

I used 0.22–0.35 g mature female medaka (more than 3 months after hatching) that had spawned eggs for at least 3 consecutive days. Medaka were deeply anesthetized with MS-222 (Sigma, St. Louis, MO). In most cases, brains were dissected and

immersed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for several hours at 4°C for fixation, and immersed in PBS containing 30% sucrose for a few hours until they sank to the bottom of the tube. After the end of the sucrose treatment, the brains were then embedded in 5% agarose (Type IX-A, Sigma) containing 20% sucrose in PBS, frozen in n-hexane at around −60°C, sectioned frontally at 10 μm on a cryostat at −25°C, and mounted onto MAS-GP–coated glass slides (Matsunami, Osaka, Japan). To detect *erα* mRNA, I used a specific DIG-labeled riboprobe which was described in chapter1. To detect *lhβ* mRNA, I also used a specific digoxigenin (DIG)-labeled riboprobe. DIG-labeled riboprobe for *lhβ* corresponding to position 3–536 of *lhβ* cDNA were generated as described previously (Kanda et al., 2011) using the DIG RNA labeling mixtures (Roche, Molecular Biochemicals GmbH, Mannheim, Germany) according to the manufacturer's protocol. All the sections were washed with PBS, and incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Then the sections were washed with PBS, and then prehybridized at 58°C for at least 30 min in hybridization buffer containing 50% formamide, 3X saline sodium citrate (SSC), 0.12 M phosphate buffer (pH 7.4), 1X Denhardt's solution (Sigma), 125 μg/ml tRNA, 0.1 mg/ml calf thymus DNA (Invitrogen, Carlsbad, CA), and 10% dextran sulfate (Sigma). The slides were incubated at 58°C overnight in the same solution containing 100 ng/ml denatured riboprobe. After hybridization, the sections were washed twice with 50 % formamide and 2X SSC for 15 min each at 58°C. Then, the sections were immersed in TNE (10 mM Tris-HCl, pH 7.5; 500 mM NaCl; and 1 mM EDTA, pH 8.0) for 10 min at 37°C. Sections were incubated with 20 μg/ml ribonuclease A (Sigma) in TNE for 30 min at 37°C, and then washed with TNE for 10 min at 37°C to remove the ribonuclease A. The sections were then washed with 2X SSC twice,

followed by 0.5X SSC twice for 15 min each at 58°C. The slides were immersed in DIG-1 (0.1 M Tris-HCl pH 8.0, 0.16 M NaCl, and 0.1% Tween 20) for 5 min, 1.5% blocking reagent with DIG-1 for 30 min, and DIG-1 for 15 min, and then incubated with an alkaline phosphatase–conjugated anti-DIG antibody (diluted 1:3,000 with DIG-1; Roche) for at least 1 h. The sections were washed with DIG-1 twice for 15 min each, and DIG-3 (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl; 0.05 M $MgCl_2$) for 3 min. The sections were then treated with a chromogenic substrate containing 337 μg/ml 4-nitroblue tetrazolium chloride (NBT) and 175 μg/ml 5-bromo-4- chloro-3-indoyl-phosphate (BCIP) in DIG-3 until a visible signal was detected. The sections were immersed in a reaction stop solution (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) to stop the chromogenic reaction. They were then dehydrated, cleared, coverslipped, and observed under light microscopy. Photographs were taken with a digital camera (DFC310FX; Leica Microsystems, Wetzlar, Germany) attached to a microscope (DM5000B; Leica Microsystems).

Double-label *in situ* hybridization analysis

I performed a double-label *in situ* hybridization by using a mixture of DIG-labeled ERα and fluorescein-labeled *lhβ* or *fshβ* probes, as described in Chapter1. *fshβ*-specific fluorescein-labeled riboprobe corresponding to nucleotide position 21–522 of *fshβ* cDNA was generated as described previously (Kanda et al., 2011) using the fluorescein RNA labeling mixtures (Roche, Molecular Biochemicals GmbH, Mannheim, Germany) according to the manufacturer's protocol. I used the same fluorescein-labeled riboprobe for *lhβ* that was generated by previous study (Kanda et al., 2011). All the procedures up to the blocking step were performed as described above. Then the sections were

incubated with a horseradish peroxidase–conjugated anti-fluorescein antibody (diluted 1:500 with DIG-1; PerkinElmer, Foster City, CA) for at least 1 h. Sections were washed twice with DIG-1 for 5 min, incubated with the Biotinyl Tyramide Amplification kit (TSA; NEL700A, PerkinElmer) (diluted 1:50 in dilution buffer; PerkinElmer) for 30 min, washed with DIG-1 twice for 5 min each, and incubated with avidin–biotin complex reagents (1% A solution and 1% B solution in DIG-1; Vector, Burlingame, CA) for 1 h. The sections were washed twice with DIG-1 for 5 min, incubated with Alexa Fluor 488–conjugated streptavidin (diluted 1:500 with DIG-1; Invitrogen) and alkaline phosphatase–conjugated anti-DIG antibody (diluted 1:1,000 with DIG-1; Roche) for 2 h. Then the sections were washed twice with DIG-1 for 5 min. After detection of *lhβ* or *fshβ* signals, the sections were incubated with DIG-3 for 5 min. Alkaline phosphatase activity was detected by using a 2-hydroxy-3-naphatonic acid-2-phenylanilide phosphate (HNPP) fluorescence detection kit (Roche) according to the manufacturer's instructions. The incubation for this substrate was carried out until visible signals were detected and was stopped by washing in PBS containing 0.5 mM EDTA. The sections were then coverslipped with CC/Mount (Diagnostic BioSystems, Pleasanton, CA). Fluorescence was observed under the confocal laser-scanning microscope LSM-710 (Carl Zeiss, Oberkochen, Germany).

Quantification of *lhβ* and *fshβ* mRNA by real time RT-PCR

The ovary was surgically removed from sexually mature females, in order to analyze effect of estrogen clearly. The fish were deeply anesthetized with 0.01% MS-222, and then the ovaries of female medaka were excised through a 2- to 3-mm incision made along the abdomen of the fish. At the end of the operation, the incision was

sutured with a nylon thread. The operated fish were allowed to survive for at least 1 week. OVX medaka were deeply anesthetized with MS-222 (Sigma). Then, I collected the pituitaries and immediately began incubation in medium [Leibovitz's L-15 medium (pH 7.4); catalog no. L5520; Sigma] at 25°C for 18 h; 10 nM 17β-estradiol (E2) was added for E2-administration group (n=5), and vehicle (0.1% ethanol) was added for control group (n=6). Given the data available for goldfish (Kobayashi et al., 1988), I expected that 10 nM E2 would be a basal level for a female teleost. After the incubation, each sample was washed with normal medium and the pituitaries were homogenized. Then, total RNA was extracted from the pituitaries using the NucleoSpin RNA XS kit (Takara, Shiga, Japan) according to the manufacture's protocol. Total RNA was reverse transcribed with a PrimeScript RT Master Mix (Perfect Real Time) kit (Takara) according to the manufacturer's instructions. Then, real-time PCR was performed as previously described (Kanda et al., 2011). The cDNA was amplified using a LightCycler 480 SYBR green I master (Roche) with the LightCycler 480 II system (Roche). The temperature profile of the reaction was 95°C for 5 min, 50 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 10 sec, and extension at 72°C for 10 sec. The PCR product was verified by the melting curve analysis. The data were normalized to a housekeeping gene, *β-actin*, and average expression levels were calculated. Primer pairs used for real-time PCR are as follows, *fshβ*-F 5′- TGGAGATCTACAGGCGTCGGTAC -3′/ *fshβ*-R 5′- AGCTCTCCACAGGGATGCTG -3′ for *fshβ*; *lhβ*-F 5′- AGGGTATGTGACTGACGGATCCAC -3′/ *lhβ*-R 5′- TGCCTTACCAAGGACCCCTTGATG -3′ for *lhβ*; and*β-actin* -F 5′- CCCCACCCAAAGTTTAG -3′/*β-actin* -R 5′- CAACGATGGAGGGAAAGACA -3′ for*β-actin*.

Data analysis

The expression levels of *fshβ* and *lhβ* in the pituitaries of estrogen (estradiol-17β) treated (E2) and vehicle (ethanol) treated group (vehicle) were analyzed using Wilcoxon rank sum test and compared with the vehicle group.

Results

Expression of *erα* mRNA in the gonadotropes

First, I analyzed the expressions of *erα* mRNA in the pituitary gland by single label *in situ* hybridization. Because the localizations of *erα* mRNA were close to the regions where gonadotropin secreting cells are expressed (Fig. 1), I analyzed possible co-expression of *erα* mRNA and *lhβ* mRNA by double-label *in situ* hybridization. I found that most *lhβ* expressing cells co-expressed *erα* mRNA (Fig. 2A).

I also analyzed the expression of *erα* mRNA in the FSH cells. I performed double *in situ* hybridization for *erα* mRNA and *fshβ* mRNA and found that most FSH cells also express ERα (Fig. 2B).

Direct effect of estrogen on the expressions of LH and FSH

I analyzed the effects of estrogen on the LH/FSH secreting cells (Fig. 3). To eliminate the effects of intrinsic gonadal steroids, I used a pituitary separated from the brain of female medaka whose ovaries had been removed surgically 7-10 days prior to the dissection. After estrogen treatment to this preparation in the culture medium, relative expression levels of *lhβ* mRNA and *fshβ* mRNA were quantified. I found that 18 hours of estrogen treatment significantly inhibited expression of *fshβ* mRNA compared

to the vehicle treatment. The expression levels of the *fshβ* mRNA of estrogen treated group was significantly lower than that of the vehicle treated group. On the other hand, the expression levels of *lhβ* were not altered significantly by the estrogen treatment. Thus, it is concluded that estrogen directly regulates the expression of *fshβ* but not that of *lhβ* mRNA in the pituitary.

Discussion

In the present Chapter, I analyzed the direct effects of estrogen on the gonadotropes. I clearly showed that both LH cells and FSH cells expressed ERα. Estrogen treatment of the pituitary preparations showed that estrogen directly regulated the expression of *fshβ*, but not *lhβ*. The results of the present Chapter suggest that estrogen exerts feedback effects on the LH cells via indirect pathway and on the FSH cells through direct pathway. It is possible that such different mechanisms enable gonadotropes to release LH/ FSH in appropriate timings in accordance with the gonadal states.

Effects of estrogen on LH cells

Results of the present Chapter showed that estrogen treatment of the whole pituitary preparations did not affect the expression of *lhβ* mRNA in medaka. On the other hand, previous study in the OVX medaka demonstrated that *in vivo* treatment of estrogen increases the expression of *lhβ* mRNA (Kanda et al., 2011) and suggested positive feedback effects of estrogen on the *lhβ* expression. Therefore, in medaka, feedback signals of the gonadal estrogen may act on LH cells indirectly through the hypothalamus and positively regulate the synthesis of LH. This hypothesis is consistent with the previous study in the black porgy (Yen et al., 2002). In the black porgy,

estrogen treatment showed different effects on the release of LH between *in vivo* and *in vitro* experiments. *in vivo* treatment of estrogen stimulates the release of LH, whereas *in vitro* treatment in dispersed pituitary cell culture showed no effect. In previous reports in teleosts, except a report in tilapia (Levavi-Sivan et al., 2006), *in vivo* treatment of estrogen up-regulates the gene expression or release of LH, Coho salmon (Dickey and Swanson, 1998), Rainbow trout (Breton et al., 1997), European sea bass (Mateos et al., 2002), European eel or Atlantic salmon (review in (Yaron et al., 2003). Thus, as suggested in medaka, it is possible that estrogen generally shows positive feedback effects on LH mainly through the hypothalamus. Further comparative analysis of the difference between *in vitro* and *in vivo* effects of estrogen on LH cells will provide important information on the feedback regulation mechanisms of the HPG axis in teleosts.

On the other hand, in mammals, *in vivo* treatment of estrogen generally down-regulates LH (review in (Shupnik, 1996)), and such negative feedback effects of estrogen on LH are considered to be mediated by hypothalamic neurons. Here, direct effects of estrogen on gonadotropes have been suggested in mammals, and it has been suggested that estrogen directly regulates the sensitivity of gonadotropes against the hypothalamic inputs, especially the GnRH1 inputs, by controlling the expressions of GnRH receptors (Gregg and Nett, 1989; Hamernik et al., 1995). In teleosts, estrogen has also been suggested to enhance the sensitivity of LH cells in response to GnRH1 (Trudeau et al., 1993). In the present Chapter, estrogen did not affect the expression of LH, but histological results showed that most of the LH cells express ERα. Although I have not elucidated how estrogen affects medaka LH cells, as in mammals or goldfish,

it is possible that estrogen enhances the sensitivity of LH cells against the hypothalamic inputs such as GnRH.

Effects of estrogen on FSH cells

The present Chapter suggested that estrogen directly acts on the FSH cells probably through the ERα and down-regulates the expression of *fshβ* mRNA independently from the hypothalamic inputs. Furthermore, it is suggested that the direct effects of estrogen on FSH cells play a major role in the negative feedback regulation on *fshβ* expression in medaka.

It thus follows that the estrogenic feedback signals regulate FSH through pathways different from those of LH. The results of the present Chapter are consistent with the previous physiological study in medaka (Karigo et al., 2014). The authors suggested that GnRH1 stimulates the release of both LH and FSH. GnRH induces rapid and transient response in LH cells, while it induces slow and long lasting Ca^{2+} response in FSH cells. Taken together with the results of the present Chapter, such different responses of LH and FSH may be due to the fact that the LH cells are more strongly regulated by the GnRH inputs, compared with FSH. It is possible that these differences in the estrogenic regulation systems for gonadotropins may enable LH/FSH to stimulate the ovarian cells at different timings.

Although the results of the estrogenic effects on FSH cells varied depending on the seasons, maturational stages, or species (Larsen and Swanson, 1997; Antonopoulou et al., 1999a; Antonopoulou et al., 1999b; Huggard-Nelson et al., 2002), results of the present Chapter provide stronger and more precise evidence for the direct effects of

estrogen on FSH by using the more intact whole pituitary preparations in which cellular interactions in the pituitary are maintained intact.

Localization of ERα in the gonadotropes

Here, I showed by double-label *in situ* hybridization that both LH cells and FSH cells express *erα* mRNA. In teleosts, *lhβ* and *fshβ* mRNA are expressed in separate cellular populations in the pituitary (Calman et al., 2001; Aizen et al., 2007; Kanda et al., 2011), whereas these two genes are expressed in the same pituitary cells in mammals (Childs. 2006). Despite such difference, both teleosts and mammals express ER in gonadotropes. These facts are consistent with the idea that *lhβ* and *fshβ* have arisen from the same ancestral gene by a gene duplication event. Recent study (Kanda et al., 2011) suggested that rearrangement of the *lhβ* gene locus of teleosts, which occurred after the divergence of teleosts and tetrapods is the reason for the different expression patterns of LH and FSH between mammals and teleosts. From the comparative syntenic analysis, Kanda and co-workers suggested that the FSH cells of the teleosts are evolutionally identical to the gonadotropes of mammals. Despite such difference in *lhβ* gene locus, results of the present Chapter showed localization of ERα in LH cells of teleost, medaka. It is possible that the steroid sensitivity of LH cells is essential character for the reproductive functions in vertebrates. Therefore, it is highly possible that estrogen sensitivity of LH cells and FSH cells has been conserved throughout vertebrates.

In conclusion, I suggest that the expression of *lhβ* is mainly up-regulated via the HPG axis, while *fshβ* is down-regulated by the direct action of gonadal estrogen. By regulations from these different systems, plasma concentrations of FSH and LH are

changed in different patterns from each other during estrus cycle and stimulate gonads to induce maturation of ovarian follicles or ovulation, respectively.

Figure legends

Fig. 1

Photomicrographs of the pituitary gland labeled by *in situ* hybridization for *erα* mRNA or *lhβ* mRNA.

A, Photographs showing the distribution of *erα* mRNA in pituitary gland of mature female medaka.

B-E, Photographs showing the distribution of *erα* or *lhβ* mRNA in adjacent sections. Note that labeling for both probes in similar cells in the pituitary. Scale bars; 100 μm.

Fig. 2

Double *in situ* hybridization for *erα* and *lhβ* or *fshβ* in the pituitary gland.

A, Photographs showing double labeling for *lhβ* mRNA (*green*) and *erα* mRNA (*magenta*) demonstrates that *lhβ*-expressing cells co-express *erα* mRNA. These results suggest that LH cells can receive estrogen through ERα.

B, Photographs showing double labeling for *fshβ* mRNA (*green*) and *erα* mRNA (*magenta*) demonstrate that *fshβ*-expressing cells co-express *erα* mRNA.

These results suggest that both LH cells and FSH cells express ERα, and directly receive estrogen signals. Scale bars; 25 μm.

Fig. 3

Distinct regulation of *lhβ* and *fshβ* gene expression by estrogen in the pituitary of mature female medaka. After incubation in L-15 medium containing estradiol (E2

group) or ethanol (vehicle group), expression of *lhβ* and *fshβ* was quantified by real-time PCR normalized by *β-actin* expression.

A, *lhβ* expression was not significantly changed by estrogen treatment.

B, *fshβ* expression was significantly down-regulated by estrogen. The vertical axes of the graphs represent relative expression levels compared to the vehicle control groups. E2, estrogen (estradiol-17β) administrated pituitary, n= 5; vehicle, ethanol administrated pituitary, n = 6. Error bars represent standard error. Data were analyzed by Wilcoxon rank sum test. **, $p < 0.01$.

Fig. 1

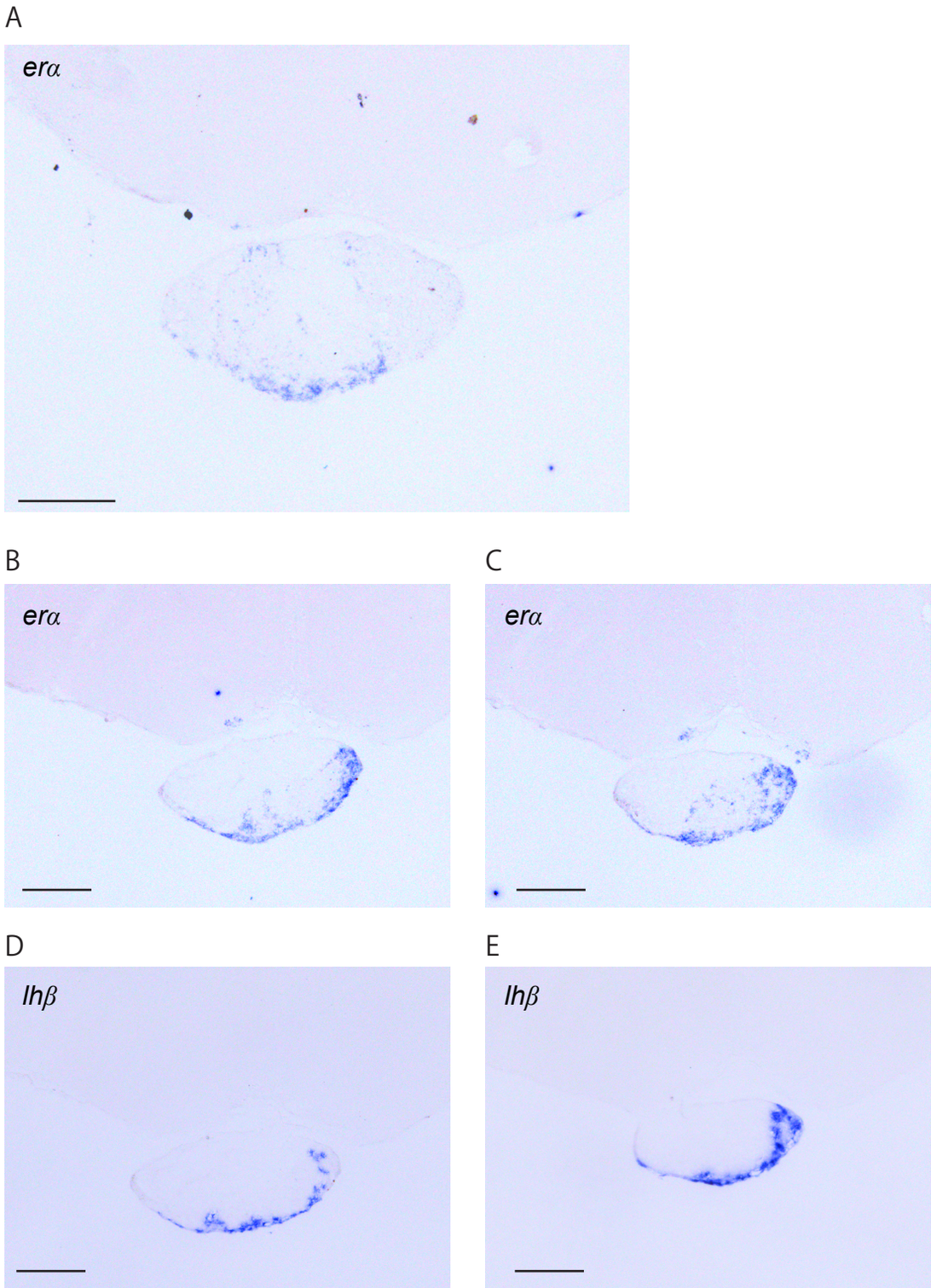
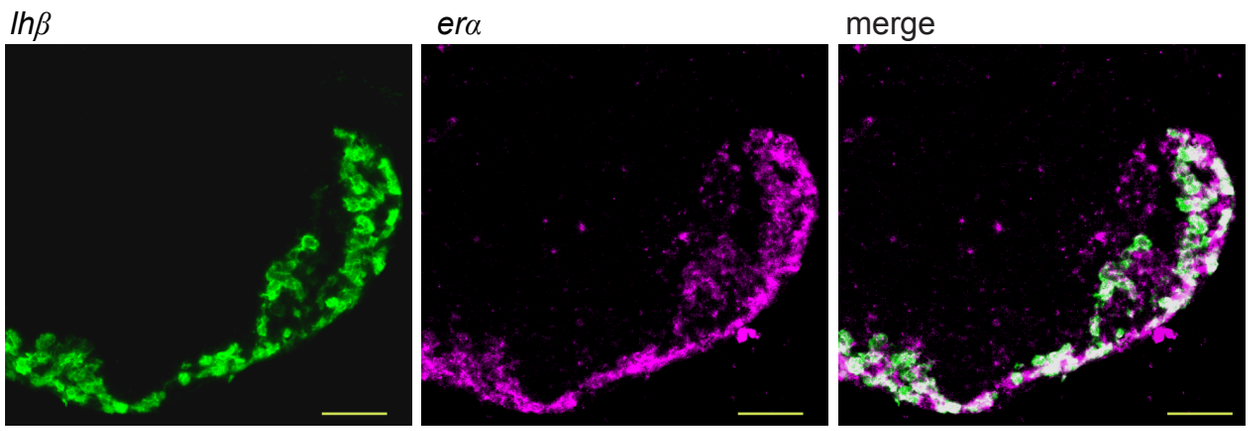


Fig. 2

A



B

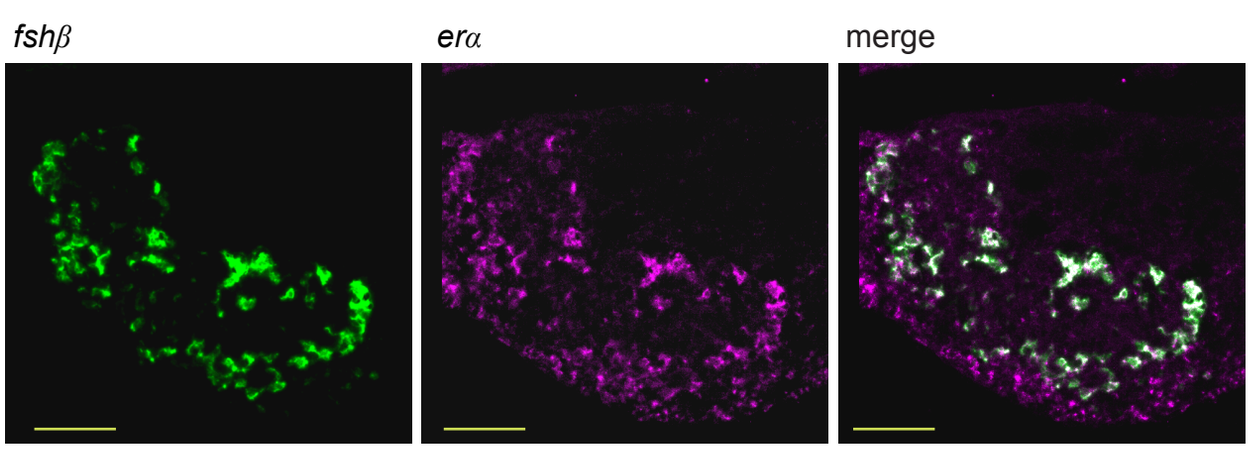
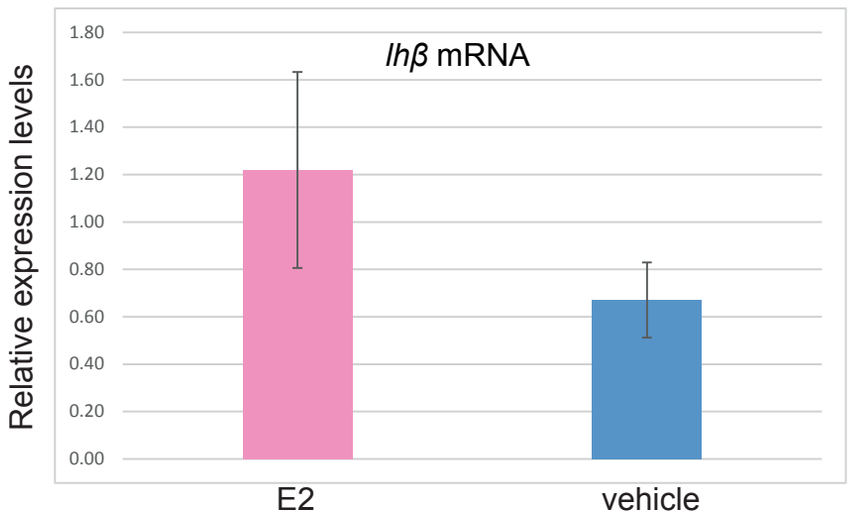
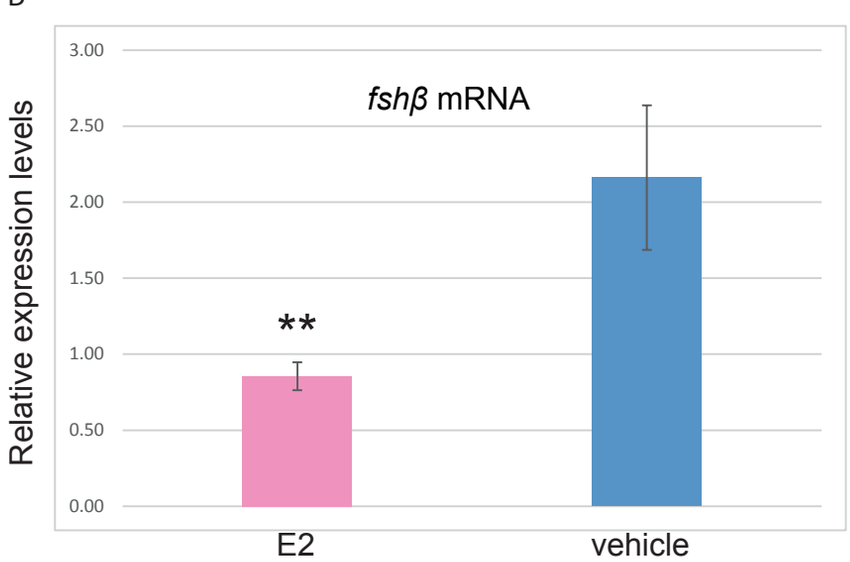


Fig. 3

A


lhβ mRNA
Relative expression levels
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
E2
vehicle

B


fshβ mRNA
Relative expression levels
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
E2
vehicle

Chapter 3

POA-ERα neurons transmit estrogen feedback signal to GnRH1 neurons for the HPG axis regulation.

Abstract

The hypothalamic neurons expressing estrogen receptor (ER) α are considered to transmit gonadal estrogen feedback signal to gonadotropin releasing hormone 1 (GnRH1) neurons, which is known as the final common pathway for the feedback regulation of reproductive functions throughout vertebrates. Moreover, most animals including vertebrates show sexual behavior after sexual maturation, especially during the breeding season, and such a regulation is also suggested to be controlled by gonadal steroids. Therefore, the neurons expressing sex steroid receptors are considered to be the immediate and important switch for these events.

For the first step towards the comprehensive understanding of how sex steroids regulate gonadotropin release and sex behaviors, I generated a transgenic (Tg) medaka line that expresses EGFP in ERα-expressing neurons (ERα neurons). By using this Tg medaka line, I analyzed the axonal projection of ERα neurons. I found that the ERα neurons in POA projected to the cell bodies of GnRH1 neurons. I also demonstrated, by double labeling *in situ* hybridization using marker genes for glutamate or gamma amino butyric acid (GABA), that most of these ERα neurons were glutamatergic, while some POA-ERα neurons were GABAergic.

Here, I hypothesize that POA glutamatergic and/or GABAergic ERα neurons relay gonadal estrogen feedback signals to GnRH1 neurons in medaka. ERα neurons receive circulating estrogen and may regulate GnRH1 neurons by glutamate and/or GABA in accordance with reproductive states, which is consistent with previous studies in mice demonstrating that glutamate/GABA synaptic transmission to GnRH1 neuron is altered by estrogen level. Taken together, I propose that the direct regulation of hypophysiotropic GnRH1 neurons mediated by glutamatergic and/or GABAergic POA-

ERα neurons is widely conserved among mammals and teleosts, and is possibly an evolutionally conserved mechanism for the feedback regulation of HPG axis throughout vertebrates. I also analyzed the projection of Vs-ERα neurons and found that they project as far as the ventral region of medulla. Taken together with results of the previous studies suggesting involvement of Vs neurons in the regulation of sexual behavior, Vs-ERα neurons are suggested to be involved in the regulation of sexual behaviors in accordance with the levels of estrogen in circulation.

Introduction

Reproduction, including sexual maturation, ovulation, sexual behavior, is produced by sophisticated interplay between the hypothalamus and the gonad. In this process, gonadal steroids are considered to play important roles not only in the feedback regulation of the hypothalamo-pituitary-gonadal (HPG) axis but also in the control of sexual behaviors. In the feedback regulation of reproductive functions, the gonads release sex steroids such as estrogen and androgen into the circulation. Sex steroids, especially estrogen, stimulate hypothalamus to release hypophysiotropic gonadotropin releasing hormone, called as GnRH1 in most animals (Okubo and Nagahama, 2008). GnRH1 acts on the pituitary and induces the release of gonadotropins; luteinizing hormone (LH) and, follicle stimulating hormone (FSH). LH and FSH, in turn, stimulate gonads and induce their maturation or ovulation. Thus, gonadal steroids play key roles in the HPG axis regulation (Christian and Moenter, 2010), and this regulatory mechanism is widely conserved in vertebrates. In addition to this feedback regulation, estrogen is also considered to play important roles in the regulation of sexual behaviors. In fact, most vertebrates show courtship behavior especially after their sexual maturation and during the breeding season. Thus, hormonal signals from gonads have been suggested to control neural circuits for the regulation of sexual behavior (Morali et al., 1977; Pfaff and Sakuma, 1979; Kim et al., 2013). In these regulatory systems of reproductive functions, gonadal estrogen can be considered as one of the most important signals on the reproductive states of the gonad to be transmitted to the central nervous system. As estrogen is known to stimulate target cells through estrogen receptor (ER)s, ERs are essential components for the brain to receive signals from gonads, and ER-expressing neurons should be considered as the first component of the neural regulatory

mechanism of reproduction. Despite such importance, the estrogen-primed neural circuits for the central regulation of reproductive functions and sexual behaviors remain to be clarified.

It has been suggested that there are two categories of ERs, ERα and ERβ and it has been strongly suggested, by using knockout mice, that ERα but not ERβ is essential for reproduction (Couse et al., 2003; Dorling et al., 2003). However, because GnRH1 neurons do not express ERα (Laflamme et al., 1998; Hrabovszky et al., 2000), GnRH1 neurons have been considered to receive estrogen feedback signal by way of other hypothalamic neurons that express ERα. Recently, in mammals, Kiss1 neurons are suggested to play crucial roles in the feedback regulation of HPG axis; 1) defects of *kiss1* or *gpr54* (Kiss1 receptor) result in infertility (de Roux et al., 2003; Seminara et al., 2003), 2) expression of *kiss1* change according to the plasma estrogen level (Smith et al., 2005), and 3) KISS1 peptide upregulates neural activity of GnRH1 neurons (Pielecka-Fortuna et al., 2008). However, in contrast to these clear results to support powerful Kisspeptin regulation on the HPG axis in mammals, there is a growing body of evidence in non-mammalian species to doubt the presence of robust regulation of Kisspeptin neurons on HPG axis. In a teleost, medaka, it has been demonstrated that GnRH1 neurons do not express Kisspeptin receptors (neither GPR54-1 nor GPR54-2, GPR54 paralogs) (Kanda et al., 2013). Recent study using *kiss1* and *kiss2* double knockout zebrafish showed normal fertility (Tang et al., 2014). Furthermore, it is known that avian species lack Kisspeptin genes (Akazome et al., 2010). These results suggest that the essential role of Kisspeptin in reproductive function, which has been suggested in mammals, are not common to all vertebrate species. Therefore, in addition to Kisspeptin neurons, it is hypothesized that some other neuronal systems also play

important roles in the HPG axis regulation in vertebrates. In the present Chapter, I tried to clarify the mechanisms of HPG axis regulation that have been widely conserved during evolution by analyzing the HPG axis regulatory mechanisms in teleosts and comparing them with those obtained in mammals.

On the other hand, neural circuits for sexual behavior have been poorly understood in most vertebrates, including mammals. Only in some teleosts, previous studies suggested that neurons in Vs region may play important role in the regulation of sexual behaviors. Electrical stimulation of Vs region in hime salmon acutely evoked male-typical sexual behavior (Satou et al., 1984). In addition, lesion study of Vs region in goldfish showed severe impairment of their sexual behaviors (Koyama et al., 1984). These results suggest that neurons in Vs region are involved in the neural circuit for sexual behavior. Vs region is also known to contain abundant ER- and androgen receptor (AR)-expressing neurons in several species (Kim et al., 1978; Zempo et al., 2013) and Chapter 1). Taken together, these previous reports suggest that estrogen modulates central regulatory system of sexual behaviors by affecting Vs region. However, neural circuits controlling sexual behaviors have not been studied precisely in any vertebrates.

To clarify the estrogen feedback pathway to the HPG axis, I have been using medaka as a model animal, because they have some unique characteristics in that they allow me various approaches for the analysis of HPG axis regulation. First, molecular genetic tools can be applied easily. Second, medaka regularly show sexual behavior every morning, and female medaka spawn daily under breeding conditions, and their breeding/nonbreeding states are controllable by changing the day length. Moreover, their pituitary is directly innervated by neurosecretory fibers and is not intercalated by

portal vessels as in other vertebrates. In addition, I have already reported on their estrogen and testosterone receptor distribution in the medaka brain (Zempo et al., 2013).

In the present Chapter, I generated a Tg medaka line that expresses EGFP in ERα neurons. This Tg medaka enables visualization of ERα neurons in live animals, which should enable various approaches to analyze their neural circuits by multidisciplinary methods; anatomical study of neural projections, transcriptome analysis of various genes (transmitters, receptors, etc.), electrophysiological analysis of ERα neurons, etc. In the present Chapter, by using this Tg line, I analyzed the neural circuit and transmitter candidates of the ERα neuron morphologically for the comprehensive understanding of how sex steroids regulate gonadotropin release and sex behaviors.

Materials and methods

Animals

Female d-rR strain medaka (*Oryzias latipes*; teleost fish) were maintained under a 14 h light, 10 h dark photoperiod at 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.

Generation of construct

Here, I generated constructs for *erα*: enhanced green fluorescein protein (EGFP) Tg medaka. I used a double-promoter approach for an efficient screening. Using the zebrafish *cardiac myosin light chain 2* (*cmlc2*) promoter, I visualized the heart for the

screening of the germline transmission. I amplified medaka genomic DNA fragment containing the 5'-flanking region (3.7 kb) of the exon2 of *erα* gene by PCR using PrimeSTAR (Takara, Shiga, Japan) from a bacterial artificial chromosome clone (BAC; clone number Golwb109_F22) of a medaka strain, HdrR, using a forward/riverse primer pair, P*erα*-F (5'-ACAGGATGGAGGTCAAAAGC-3') /P*erα*-R (GACCCCCTCGGTGACATGTATCCACCGGTCGCCACCATGG-3'). P*erα*-F (5′-CAGAACTTCCTTGCTCATGCTCACC-3′)/P*erα*-R LINKEGFPhind (5′-GAGAAGCTTCAGAGCCCTTCCCCTGTGCTCAGGC-3′). Then, I fused this amplicon with an EGFP open reading frame by overlap extension PCR. Downstream of the coding sequence of EGFP, I fused *cmlc2* promoter region and I-SceI restriction site. The total approximately 6-kb fragment was cloned into the TOPO-XL cloning vector (Invitrogen, Carlsbad, CA). The following microinjection and screening steps have been described previously (Okubo et al., 2006). After generation of the transgenic line, to confirm the specific EGFP expression in ERα neurons, I performed double labeling of *erα* mRNA *in situ* hybridization and EGFP immunohistochemistry using cryostat brain sections of this transgenic line. Methods of double labeling of IHC and ISH are described in the next paragraph.

Double labeling for EGFP and mRNA of *erα*, *gnrh1*, *lhβ*, or *fshβ*

The brain of *erα*:EGFP transgenic line was fixed by 4% PFA in PBS. The fixed brain was frontally cryosectioned at 20 μm using a cryostat (CM 3050S; Leica Microsystems) and mounted onto MAS-GP type A coated glass slides (Matsunami, Osaka, Japan). The sections were incubated with anti-EGFP antibody raised in rabbit (generous gift from Drs. Kaneko and Hioki, Kyoto University, Kyoto, Japan diluted,

1:1000; with PBS containing 0.3% Tween 20 (PBST]) for overnight, rinsed twice with PBST, and incubated with butylated antirabbit IgG (Invitrogen) (diluted 1:200 with PBST) for 2h. Then, sections were fixed by 4% PFA in PBS 15 min, and rinsed with PBS containing 0.2% glycine and PBS.

Then, I performed *in situ* hybridization using these slides, to detect *erα*, *gnrh1*, *lhβ*, or *fshβ* mRNA. I used *erα*-, *lhβ*-, or *fshβ*-specific DIG-labeled probe that were described in Chapter1 and Chapter2. I used *gnrh1*-specific DIG-labeled probe that was generated previously (Karigo et al., 2012), and performed *in situ* hybridization as described in Chapter1. The slides were incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Then the sections were washed with PBS, and then prehybridized at 58°C for at least 30 min in hybridization buffer containing 50% formamide, 3X saline sodium citrate (SSC), 0.12 M phosphate buffer (pH 7.4), 1X Denhardt's solution (Sigma, St. Louis, MO), 125 μg/ml tRNA, 0.1 mg/ml calf thymus DNA (Invitrogen), and 10% dextran sulfate (Sigma). The slides were incubated at 58°C overnight in the same solution containing 100 ng/ml denatured riboprobe. After hybridization, the sections were washed twice with 50 % formamide and 2X SSC for 15 min each at 58°C. Then, the sections were immersed in TNE (10 mM Tris-HCl, pH 7.5; 500 mM NaCl; and 1 mM EDTA, pH 8.0) for 10 min at 37°C. Sections were incubated with 20 μg/ml ribonuclease A (Sigma) in TNE for 30 min at 37°C, and then washed with TNE for 10 min at 37°C to remove the ribonuclease A. The sections were then washed with 2X SSC twice, followed with 0.5X SSC twice for 15 min each at 58°C. The slides were immersed in DIG-1 (0.1 M Tris-HCl pH 7.5, 0.16 M NaCl, and 0.1% Tween 20) for 5 min, 1.5% blocking reagent with DIG-1 for 30 min, and DIG-1 for 15 min, and then incubated with incubated with avidin–biotin complex (ABC) reagents

(1% A solution and 1% B solution in DIG-1 buffer; Vector Laboratories, Burlingame, CA) for 1 h. The sections were rinsed twice with DIG-1 buffer, incubated with Alexa Fluor 488-conjugated streptavidin (diluted 1:500 with DIG-1 buffer; Invitrogen) and alkaline phosphatase-conjugated anti-DIG antibody (diluted 1:1000 with DIG-1 buffer; Roche, Molecular Biochemicals GmbH, Mannheim, Germany) for 2 h. Then, the sections were rinsed twice with DIG-1 buffer. After the EGFP signals were detected, the alkaline phosphatase activity, which was used to label mRNA, was detected using Fast-Red substrate kit (Roche) according to the manufacturer's instructions. The incubation for this substrate was carried out until visible signals were detected and was stopped by washing in PBS containing 0.5 mM EDTA. Then, the sections were cover slipped with CC/Mount (Diagnostic BioSystems). The fluorescence was observed under confocal laser-scanning microscope LSM-710 (Carl Zeiss, Oberkochen, Germany) at 1-μm optical section. I calculated percentage of ventro-lateral GnRH1 neurons which receive projections from EGPF fibers, by counting cell bodies of GnRH1 neurons that were surrounded by EGFP labeled fibers within 1μm.

Immunohistochemistry (IHC) for tract tracing

I carried out Immunohistochemistry (IHC) for EGFP to analyze neural projections of ERα neurons. All the procedures up to the secondary anti-body, biotinylated anti-rabbit IgG (Invitrogen), were performed as described above. The sections were washed twice with PBST, and then incubated with ABC reagents (1% A solution and 1% B solution in PBST buffer; Vector) for 1 h. Then, slides were washed twice with PBST, and reacted with 3, 3’-diaminobenzidine. For some slides, I performed Nissl staining. The sections were counter-stained with 0.1% cresyl violet solution. Then, slides were

dehydrated, cleared, and coverslipped. Photographs were taken with a digital camera (DFC310FX; Leica Microsystems, Wetzlar, Germany) attached to a microscope (DM5000B; Leica Microsystems).

Innervation of the pituitary by POA-ERα neurons was examined using a dual fluorescence method by retrograde labeling of neurobiotin and EGFP immunohistochemistry as described previously (Karigo et al., 2012). Briefly, I anesthetized fish and dissected out the whole brain and pituitary of female *erα*:EGFP Tg medaka, and then inserted a small crystal of neurobiotin (Sigma) into the pituitary. After incubation of the brain in fish artificial cerebrospinal fluid (ACSF) containing, 134 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES pH 7.4, and 15 mM glucose for 120 min, brains were fixed with 4% PFA in PBS. The fixed brain was sectioned frontally at 30 μm using a cryostat (CM 3050S; Leica Microsystems, Wetzlar, Germany) and mounted onto MAS-GP type A-coated glass slides (Matsunami). Then, the retrogradely labeled neurobiotin signals were visualized with Alexa Fluor 488-conjugated streptavidin (1:500; Invitrogen) by reaction with ABC Elite kit (Vector). EGFP was visualized by anti-EGFP antibody and Alexa Fluor 555-conjugated anti-rabbit IgG (1:800; Invitrogen). The incubation for this IgG was carried out until visible signals were detected and was stopped by washing in PBS. The sections were then coverslipped with CC/Mount (Diagnostic BioSystems). The fluorescent signals were observed using a LSM-710 confocal laser-scanning microscope (Carl Zeiss).

Double-label *in situ* hybridization for *erα* mRNA and *vglut* or *gad* mRNA

I performed a double-label *in situ* hybridization by using a mixture of DIG-labeled *erα* and fluorescein-labeled *vesicular glutamate transporter (vglut) 2.1*, *glutamic acid decarboxylase (gad)1.1*, *gad1.2* and *gad2* probes that were synthesized based on the previous report (Akazome et al., 2011). Medaka-cDNAs were PCR-amplified using a forward/riverse primer pairs,
vglut2.1-F 5'-GAGATCAACCTGCGCTCACCACA-3'/ *Vglut2.1*-R 5'-TGAATACTGAACCAGGATCCCAG-3',
gad1.1-F 5'-GAGGCTGTGACTCATGCGTG-3'/ *gad1.1*-R 5'-CCTTCTTTATGGAATAGTGGC-3',
gad1.2-F 5'-GCCAGATCCACGCTGGTGGAC-3'/ *gad1.2*-R 5'-CATTAGCACAAAAACTGGAG-3',
gad2-F 5'-AAACAGCCCATCCCAGGTAC-3'/ *gad2*-R 5'-AGCAGCGGGATTTGAGATGAC-3', and then, these amplified fragments were used to generate probes using the fluorescein RNA labeling mixtures (Roche) according to the manufacturer's protocol.

All the procedures up to the blocking step were performed as described above (brains were fixed by transcardial perfusion). Then the sections were incubated with a horseradish peroxidase conjugated anti-fluorescein antibody (diluted 1:500 with DIG-1; PerkinElmer, Foster City, CA) for at least 1 h. Sections were washed twice with DIG-1 for 10 min, incubated with the Biotinyl Tyramide Amplification kit (TSA; NEL700A, PerkinElmer) (diluted 1:50 in dilution buffer; PerkinElmer) for 30 min, washed with DIG-1 twice for 10 min each, and incubated with ABC reagents (Vector) for 1 h. The sections were washed twice with DIG-1 for 10 min, incubated with Alexa Fluor 488–conjugated streptavidin (diluted 1:500 with DIG-1) and alkaline phosphatase conjugated

anti-DIG antibody (diluted 1:1,000 with DIG-1) for 2 h. Then the sections were washed twice with DIG-1 for 10 min. After detection of positive signals, alkaline phosphatase activity was detected by using Fast-Red substrate kit (Roche) according to the manufacturer's instructions. The incubation for this substrate was carried out until visible signals were detected and was stopped by washing in PBS containing 0.5 mM EDTA. The sections were then coverslipped with CC/Mount (Diagnostic BioSystem). The fluorescent signals were observed using a LSM-710 confocal laser-scanning microscope (Carl Zeiss).

Results

Establishment of *erα*:EGFP transgenic line

I generated a transgenic medaka line that expresses EGFP in ERα neurons specifically. I found that the EGFP positive cells were mainly distributed in the Vs, POA, and NVT region, all of which have previously been reported to contain ER expressing cells (Zempo et al., 2013). In the present Chapter, I use the term POA as representing the rostral part of POA (called rPOp in Chapter1).

For the examination of specificity, I carried out double labeling analysis of EGFP immunohistochemistry and *erα* mRNA *in situ* hybridization (Fig. 1). It was shown that 44% of POA-ERα neurons (n=3), and about 70% of Vs-ERα neurons (n=2) and NVT-ERα neurons (n=2) were labeled with anti-EGFP antibody. Although there were some EGFP negative ERα cells in these areas, all the EGFP positive cells expressed ERα, at least in these areas. Thus, the specificity of EGFP labeling should be satisfactory for performing anatomical analysis of axonal projections (in this Chapter) and future electrophysiological studies.

Anatomical study of the axonal projections of the ERα neurons

I performed EGFP immunohistochemically in the brain of *erα*:EGFP transgenic medaka to analyze neuronal projections of ERα neurons. Immunohistochemistry for EGFP, which enhances EGFP signals, enabled precise morphological analysis of axonal projections. Most POA-ERα neurons projected their axons to the lateral region of POA. These fibers formed a thick bundle and continued caudally (Fig. 2 B). Frontal sections show that fibers project caudally, passing through the ventro-lateral region of telencephalon and hypothalamus (Fig. 2 C). In the hypothalamus, the axons of the POA-ERα neurons coursed laterally to the NAT and NVT. These bundles of fibers innervated the pituitary. Although these results do not exclude another possibility that some POA-ERα neurons project to the other regions, the majority of axons of the POA-ERα neurons projected to the pituitary. Immunohistochemistry for EGFP shows the distribution of the EGFP-labeled axons of the POA-ERα neurons in the pituitary gland (Fig. 3). The axons of the ERα neurons entered the rostral pituitary and widely projected in the rostral half of the pituitary, whereas few EGFP immunoreactive fibers were observed in the caudal region. Especially in the region where LH and FSH cells are localized (midway along the rostrocaudal axis of the pituitary), EGFP positive fibers were distributed abundantly.

Then, to examine if these axons in the pituitary originate from POA-ERα neurons, a retrograde axonal tracer neurobiotin was injected in the pituitary. POA-ERα neurons were double-labeled by EGFP and retrogradely transported neurobiotin (Fig. 4). Thus, POA-ERα neurons were proven to directly innervate the pituitary gland.

Analysis of the sagittal sections showed that the Vs-ERα neurons mainly projected in the caudal regions of the brain (Figs. 5 A and B). Analysis of the frontal sections showed that these EGFP positive fibers projected caudally, passing near the midline region of the hypothalamus bilaterally. These axons passed through the periventricular region of the hypothalamus, in the region lateral to Nppv, and coursed further caudally. Finally, some axons reached the ventromedial region of medulla and terminated there. The axons of Vs-ERα neurons terminated in the region ventral to RFM (Fig. 5 C). In this Tg line, there were no axons of Vs-ERα neurons that projected to the spinal cord.

Morphological analysis of the axonal projections of ERα neurons to the HPG axis components (GnRH1 neurons and LH/FSH cells)

To assess the participation of ERα neurons in mediating the estrogen actions on the GnRH1 neurons, which is generally suggested to be the most important part of gonadotropin release regulation in the hypothalamus, I performed double labeling analysis of EGFP IHC and *gnrh1* mRNA ISH. The EGFP positive axons from the POA-ERα neurons surrounded the GnRH1-expressing neuronal cell bodies localized in the ventrolateral part of POA (Fig. 6). 77% of GnRH1 neurons received axons of the POA-ERα neurons (n=3). On the other hand, few axons were observed around the dorsal and medial cell groups of GnRH1 neurons, which have not been suggested to project to the pituitary (Fig. 6 C).

I also examined the innervation of ERα neurons to the pituitary gonadotropin-producing cells (LH and FSH cells) by double labeling analysis of EGFP IHC and *lhβ* mRNA or *fshβ* mRNA ISH. The axons of ERα neurons were observed in the deep layer of LH cells. The merged image shows that axons of the ERα neurons directly contact

some LH cells (Fig. 7 A). On the other hand, axons of the ERα neurons surrounded the FSH cells and appeared to contact some FSH cells directly (Fig. 7 B). It should be noted, however, that axons of the ERα neurons were distributed rather broadly in the rostral half of the pituitary gland, which may suggest that ERα neurons also regulate release of hormones other than gonadotropins.

Neurotransmitter candidates for the POA-ERα neurons

To identify the neurotransmitter candidates for the POA-ERα neurons, I analyzed co-localization of glutamate or GABA, typical classical transmitters in the brain, in the POA-ERα neurons by double *in situ* hybridization for the mRNA of the marker genes for glutamatergic and GABAergic neuron, *vglut2.1* and *gad1.1*, respectively with *erα* mRNA.

Almost all of the POA-ERα neurons co-expressed *vglut 2.1* (Fig. 8 B). In addition, some POA-ERα neurons co-expressed *gad 1.1* (Fig. 8 C). In addition to these genes, it has been reported that medaka possess 4 subtypes of *vglut* and 2 subtypes of *gad* (Akazome et al., 2011). As most ERα neuron were shown here to be glutamatergic by double labeling of ERα and *vglut 2.1*, I did not examine other subtypes of *vglut*. For GABA, amongst all GABA synthetic enzymes, *gad1.1* showed the highest percentage of co-localization with ERα mRNA (Figs. 8 and 9). However, the co-localization ratio for *gad1.1* was much lower than that of *vglut2.1*, which suggests that the main classical neurotransmitter in the POA-ERα neurons is glutamate.

Discussion

Although estrogen has been considered as a key regulator of HPG axis as well as sexual behaviors, most of the previous studies have been focused on the elucidation of anatomy and physiology of neuronal systems that are considered to be essential for the estrogen-primed regulation of reproductive functions, e.g., GnRH and Kisspeptin neuronal systems. In the present Chapter, I tried to approach the problem from quite different aspects. First, I established an EGFP transgenic medaka line for specific visualization of neurons *in vivo*, which enabled the detailed anatomical analysis of the axonal projections of the ERα neurons (present Chapter) and should also prove to be invaluable for future physiological analyses of intact ERα neurons using the whole brain *in vitro* preparations (Karigo et al., 2012). Because EGFP passively diffuses throughout the axon and dendrites, the projection of ERα neurons could be easily visualized in the present Chapter. For the first step towards understanding the estrogenic regulation of neurons in the brain, I investigated the projection of ERα neurons to the HPG axis components, the POA GnRH1 neurons and the pituitary gonadotropes. In addition, I also analyzed the projection of Vs-ERα neurons because of their possible involvement in the regulation of sexual behavior.

Specificity of EGFP labeling in the brain of *erα*:EGFP transgenic medaka line

In the brain of *erα*:EGFP transgenic medaka, I found that EGFP signals were distributed in POA, Vs, NVT, and I could confirm the specificity of EGFP labeling in these regions (see Results).

On the other hand, nonspecific EGFP labeling was found in NDTL and optic tectum, where *erα* mRNA was not detected. However, these labeling was rather local and did

not interfere with the analysis of axonal projections of the POA or Vs neurons in the present Chapter.

Functional considerations of Vs-ERα neurons

In the present Chapter, I could provide novel insights into the neural circuit underlying regulation of sexual behavior mediated by estrogen. In the previous studies, effects of estrogen on sexual behavior have been analyzed by estrogen implantation in the brain. For example, in rodents, estrogen was suggested to activate lordosis of female rats (Pfaff and Sakuma, 1979). In teleosts, female guppy, ovariectomy inactivates sexual behaviors, and they can be restored by estrogen treatments (Liley, 1972). Although these previous reports suggested that estrogen is involved in the regulation of sexual behavior, the action site of estrogen in the neural circuit of sexual behavior has not been clarified so far, because the component of neural circuit of sexual behavior has not been identified precisely in any vertebrates.

In some teleosts, previous studies reported that Vs is an important component of the neural circuit of sexual behavior. Electrical stimulation of Vs acutely induced sexual behavior in hime salmon (Satou et al., 1984). Such acute response may suggest that neurons in Vs are involved in the regulation of motor aspects of the sexual behavior. However, neural pathway of neurons in Vs has not been studied in detail. Here, the results of Chapter 1 indicates that neurons expressing all three subtypes of ER are distributed in Vs of the medaka brain, suggesting that the neural circuit of sexual behavior are possibly modulated by estrogen through ER neurons in Vs region, although their projection remain to be elucidated. In the present Chapter, I showed that Vs-ERα neurons project as far as the ventral region of medulla. However, I did not find neural

fibers of Vs-ERα neurons in the spinal cord in the present Tg line Thus, Vs-ERα neurons are not considered to stimulate motor neuron controlling sexual behavior directly. It is therefore possible that gonadal estrogen acts on the Vs-ERα neurons and possibly regulates expression of neurotransmitters or firing activity, in accordance with the gonadal states. Uncharacterized neurons in the medulla that receive axonal projections from the Vs-ERα neurons may relay this estrogenic modulation to the motor pattern generator in the spinal cord to induce sexual behavior. Recent study in medaka has suggested that neuropeptide B (NPB) is female specifically expressed in Vs-ERα neurons, and the expression of NPB is up-regulated by estrogen (Hiraki et al., 2014). It can be considered that Vs-NPB/ERα neurons is involved in the regulation of female-typical sexual behavior.

Further analyses of both physiological and morphological properties of Vs-ERα neuron as well as characterization of the medullary neurons should provide a key to understanding the neuronal circuits underlying estrogen-primed modulation of sexual behavior.

Functional considerations of POA-ERα neurons

In the present Chapter, I suggested a novel possibility that POA-ERα neurons play a key role in the feedback regulation system of HPG axis, by regulating GnRH1 neuron directly. POA-ERα neurons were suggested to mainly project to the ventrolateral group of GnRH1 neurons but not to the other groups, medial and dorsal cell groups of GnRH1 neurons. In medaka brain, GnRH1 neurons can be classified into three different populations. Previous studies already reported that only the ventrolateral GnRH1 neurons project to the pituitary and regulate LH/FSH release, whereas the other two

populations project to the other brain areas (Karigo et al., 2012; Okubo et al., 2006). Therefore, the POA-ERα neurons are considered to regulate the hypophysiotropic GnRH1 neurons (the ventrolateral group of GnRH1 neurons) directly. In my double ISH study (**Neurotransmitter candidates for the POA-ERα neurons**), I demonstrated that most of these ERα neurons were glutamatergic, while some POA-ERα neurons were GABAergic. Taken together with the above mentioned results of the axonal projections, it is strongly suggested that glutamatergic/GABAergic POA-ERα neurons directly regulate hypophysiotropic ventrolateral GnRH1 neurons to regulate the HPG axis.

On the other hand, in mammals, Kiss1 neurons have recently been suggested to stimulate GnRH1 neurons in accordance with estrogen level (de Roux et al., 2003; Seminara et al., 2003; Pielecka-Fortuna et al., 2008). On the other hand, several previous reports in teleosts have suggested that either Kiss1 or Kiss2 (product of paralogous gene of *kiss1*) neurons show estrogen sensitivity in the brain (Kanda et al., 2008; Kanda et al., 2012). In medaka brain, Kiss1 neurons are localized in NVT region, and expression of *kiss1* mRNA is up-regulated by estrogen (Kanda et al., 2008). Thus, steroid sensitivity of Kiss1 neuron is common to mammals and teleosts. However, in teleosts, Kiss1 neuron do not act on GnRH1 neuron directly. In medaka, GnRH1 neurons lack Kiss1 receptor, GPR54 (Kanda et al., 2013). Recent *kiss1* knockout study in zebrafish showed normal fertility (Tang et al., 2014). Furthermore, avian species do not possess genes for Kisspeptins nor GPR54 (Akazome et al., 2010), and no strong evidence has been reported to suggest the presence of Kiss1-mediated HPG axis regulation in non-mammalian species. Thus, direct regulation of GnRH1 neurons by Kiss1 neurons may not be a common feature throughout vertebrates.

In contrast, the neural circuit suggested in the present Chapter is consistent with that previously reported in mice, which demonstrated that glutamate/GABA synaptic transmission to GnRH1 neuron is altered by estrogen levels (Christian and Moenter, 2007; Christian et al., 2009). In addition, there are some reports suggesting that the percentage of glutamate responsive GnRH1 neurons is changed across puberty; the response of GnRH1 neuron to GABA is also changed across puberty (Terasawa and Fernandez, 2001; Herbison and Moenter, 2011). Moreover, infusion of antagonist of glutamate receptor or GABA receptor induced precocious puberty in mammals. From these previous studies, glutamate/GABA has been considered to play important roles in the regulation of HPG axis and puberty onset in mammals (Terasawa and Fernandez, 2001). In the present Chapter, I demonstrated for the first time in vertebrates that many POA glutamatergic neurons express *erα* mRNA and project to the hypophysiotropic GnRH1 neurons. Taken together with results of the above mentioned mammalian physiological studies, I propose that the direct regulation of hypophysiotropic GnRH1 neurons mediated by glutamatergic and/or GABAergic POA-ERα neurons is widely conserved among mammals and teleosts, and is possibly an evolutionally conserved mechanism for the feedback regulation of HPG axis throughout vertebrates.

However, there still remains a possibility that POA-ERα neurons co-express neuropeptide(s) in addition to glutamate and GABA, and the neuropeptide play an important role in the regulation of GnRH1 neurons. Further analysis such as transcriptome analysis should be targeted to the POA-ERα neurons to extend our understanding of the central regulatory mechanisms of HPG axis.

Functional considerations for the axons in the pituitary gland originating from the POA-ERα neurons

Results of the present anatomical analysis suggested that POA-ERα neurons may directly regulate gonadotropes. However, it should be noted that there still remains a possibility that neuronal fibers originating from the POA-ERα neurons regulate the release of pituitary hormones other than LH or FSH. In spite of this, GnRH1 has been considered as the most powerful and essential regulator of gonadotropin release, because hypophysiotropic GnRH deficiency has been suggested to cause infertility (Cattanach et al., 1977), and the HPG axis has been considered as the general mechanism for the regulation of reproductive functions in vertebrates (Childs, 2006; Karigo and Oka, 2013). Further investigation using this transgenic line may unveil a novel HPG axis-independent neuronal pathway of gonadotropin release regulation mediated by the POA-ERα neurons directly projecting to the pituitary.

In conclusion, in the present Chapter, I propose three main neuronal pathways that relay estrogen action to the brain and regulate neuronal functions related to reproduction and sexual behaviors; two pathways for regulating reproduction, the POA-ERα neurons-pituitary and the POA-ERα neurons-GnRH1 neurons pathways, and one pathway for regulating sexual behavior, the Vs-ERα neurons-medulla pathway.

Figure legends

Fig. 1

Establishment of *erα*:EGFP transgenic medaka.

A, The construct used to generate the *erα*:EGFP transgenic medaka. The EGFP-coding sequence was fused to the 3.7-kb DNA fragment containing the 5'-flanking region of exon1, intron1, and part of exon2 of the *erα* gene. This construct contains the *cardiac myosin light chain 2* (*cmlc2*) promoter region to express EGFP in the heart for screening, and also contains the I-SceI restriction site.

B, Illustrations of a lateral view (*left*, rostral) and a frontal section of the medaka brain, showing the plane of section corresponding to the panels in C. *Red square* indicates the preoptic area (POA) population of ERα neurons shown in C; note that midline is located in the center of the pictures in C.

C, Photographs showing double labeling for EGFP immunohistochemistry (*green*) and *erα* mRNA (*magenta*) in POA region demonstrates that EGFP specifically label ERα neurons. In addition to POA, I confirmed specificity of EGFP labeling in supracommissural part of ventral telencephalic area (Vs) and nucleus ventralis tuberis (NVT) region, which are suggested to express ERα (data not shown). Scale bars; 10μm.

Fig. 2

Light photomicrographs showing the sagittal (A, B) and frontal (C) sections of the *erα*:EGFP neurons visualized with anti-EGFP antibody in the brain of female *erα*:EGFP transgenic medaka. Sections were counter-stained with cresyl violet.

A, Red square in the schematic illustration (lateral view of the brain) indicates the area corresponding to photographs in panel B.

B, Sagittal sections showing EGFP immunoreactive cell bodies in POA (arrow head) and fibers (arrow). Photographs showing different medial-lateral levels of the brain. Most fibers of the POA-ERα neurons project caudally and reach ventral region of hypothalamus.

C, Frontal sections showing axons of the ERα neurons. Illustrations of a frontal section of the medaka brain, showing the planes of section corresponding to the photographs. Black squares indicate the areas corresponding to the photographs. POA-ERα neurons project their axons to the lateral region of POA and run caudally, passing the ventrolateral region of hypothalamus, and project to the pituitary (pit). Scale bars; 100μm.

Fig. 3

Light photomicrographs of the frontal sections of the pituitary of female *erα*:EGFP transgenic medaka showing axons originating from the *erα*:EGFP neurons visualized with anti-EGFP antibody in the brain. Photographs show different rostro-caudal levels of the pituitary. The axons were abundantly distributed in the midway along the rostrocaudal axis of the pituitary (pit), whereas there were few axons in the caudal region. Scale bars; 100μm.

Fig. 4

Double labeling analysis suggests that POA-ERα neurons directly project to the pituitary.

A, Illustration of a frontal section of the medaka brain, showing the plane of section corresponding to the panels in B. Red square indicates the area corresponding to the panels in (B).

B, Double fluorescence analysis with retrograde labeling of neurons after neurobiotin injection to the pituitary of *erα*:EGFP transgenic medaka (B, magenta) and EGFP immunohistochemistry (B, green), demonstrating that the POA-ERα neurons project directly to the pituitary (B). White arrowheads in photographs indicate neurons that show co-localization of retrograde tracer and EGFP, showing projections of the axons of POA-ERα neurons to the pituitary. Scale bars, 25 μm.

Fig. 5

Light photomicrographs showing the sagittal (A, B) and frontal (C) sections of the *erα*-EGFP neurons visualized with EGFP antibody in the brain of female *erα*:EGFP transgenic medaka. The sections were counter-stained with cresyl violet.

A, Sagittal section showing EGFP immunoreactive (ir) cell bodies and axons. Red square in the schematic illustration (lateral view of the brain) indicates the area corresponding to the photograph.

Arrowheads indicate cell bodies in Vs region. I found that EGFP-ir neurons in Vs (Vs-EGFP-ir neurons) project their axons to the caudal region.

B, Sagittal sections more lateral to the photograph in (A) showing that axons from Vs region project caudally and, which suggest that these fibers project to the medulla, passing through the diencephalon. Arrows indicate fibers that originate from Vs-ERα neurons.

C, Frontal section showing fibers of the Vs EGFP-ir neurons in the ventral region of medulla. Arrowheads indicate EGFP-ir axons. EGFP-ir fiber was not observed in more caudal regions. These results suggest that the Vs-ERα neurons project caudally and terminate in the ventral region of medulla. Scale bars; 100μm (A, B), 50μm (C).

Fig. 6

Double labeling analysis using *erα*:EGFP transgenic medaka suggests that POA-ERα neurons directly contact the cell body of the GnRH1 neuron.

A, Illustration of a frontal section of the medaka brain, showing the plane of section corresponding to the panels in B. Red square indicates the area corresponding to the panels in B and C.

B, Photographs showing double labeling EGFP immunohistochemistry (*green*) and *gnrh1* mRNA *in situ* hybridization (*magenta*) demonstrate that cell body of GnRH1 neurons are surrounded by EGFP positive fibers. Taken together with the distribution of axons of ERα neurons, the POA-ERα neurons are suggested to make direct contacts on the GnRH1 neurons.

C, Double fluorescence EGFP immunohistochemistry (*green*) and *gnrh1* mRNA *in situ* hybridization (*magenta*) demonstrate that not all GnRH1 neurons receive POA-ERα neurons. There was no axons around the medial cell group of GnRH1 neurons (white arrowhead). The POA-ERα neurons projected to the ventrolateral group of GnRH1 neurons, which are suggested as a hypophysiotropic cell group for the regulation of HPG axis. Scale bars; 20μm.

Fig. 7

Double labeling analysis for *erα*:EGFP transgenic medaka suggests that POA-ERα neurons project to the LH cells and FSH cells.

A, Photographs showing double labeling for EGFP immunohistochemistry (*green*) and *lhβ* mRNA *in situ* hybridization (*magenta*) suggest that LH cells receive EGFP positive fibers.

B, Photographs showing double labeling for EGFP immunohistochemistry (*green*) and *fshβ* mRNA *in situ* hybridization (*magenta*) demonstrate that FSH cells are surrounded by EGFP positive fibers. Scale bars; 25μm.

Fig. 8

Double *in situ* hybridization for *erα* and marker genes for glutamate or GABA in POA.

A, Illustration of a frontal section of the medaka brain, showing the plane of section corresponding to the photographs. Red square indicates the area corresponding to the photograph in B and C.

B, Photographs showing double labeling for *vglut2.1* mRNA (*green*) and *erα* mRNA (*magenta*) demonstrate that POA-ERα expressing cells co-express *vglut2.1*. These results suggest that most of the POA-ERα neurons are glutamatergic.

C, Photographs showing double labeling for *gad1.1* mRNA (*green*) and *erα* mRNA (*magenta*) demonstrate that some POA-ERα expressing cells co-express *gad1.1*. About 30-40% of POA-ERα expressing neurons are suggested to co-express GABA (n=2). These results suggest that most POA-ERα expressing neurons produce glutamate, and some POA-ERα expressing neurons produce GABA. Scale bars; 25μm.

Fig. 9

In situ hybridization for *erα* and marker genes of GABA in POA region.

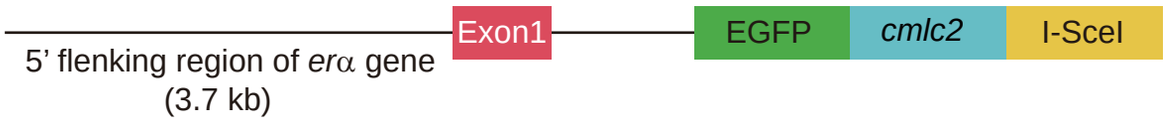
A, Photographs showing merged image of double *in situ* hybridization for *gad1.2* mRNA (*green*) and *erα* mRNA (*magenta*) demonstrate that very few POA-ERα cells co-express *gad1.2.*

B, Photographs showing merged image of double *in situ* hybridization for *gad2* mRNA (*green*) and *erα* mRNA (*magenta*) demonstrate that some POA-ERα cells co-express *gad1.2.*

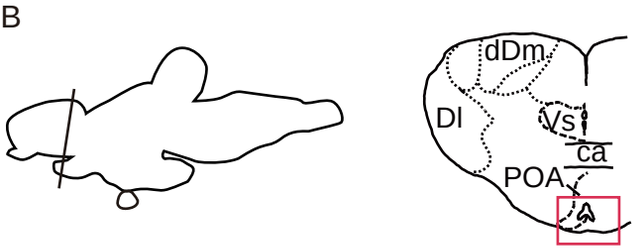
Taken together with Fig. 8, GABAergic POA-ERα expressing neuron mainly expressed *gad1.1*. For GABA, amongst all GABA synthetic enzymes, *gad1.1* showed the highest percentage of co-localization with *erα* mRNA (Fig. 8). Scale bars; 20μm (A) and 25μm (B).

Fig. 1

A


Exon1
EGFP
cmlc2
I-SceI
5’ flenking region of erα gene
(3.7 kb)

B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Dl
Vs
ca
POA

C

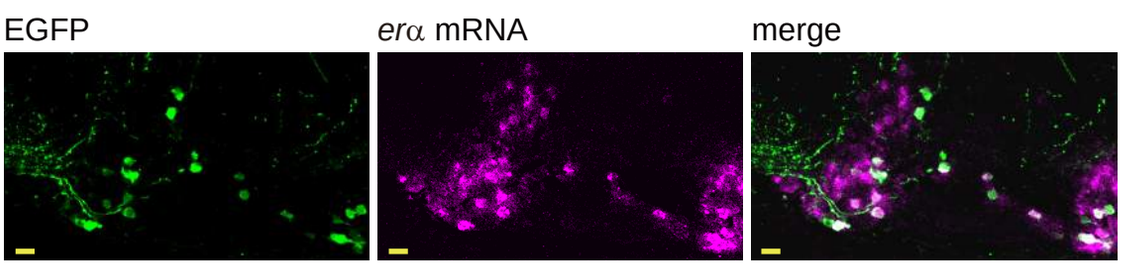
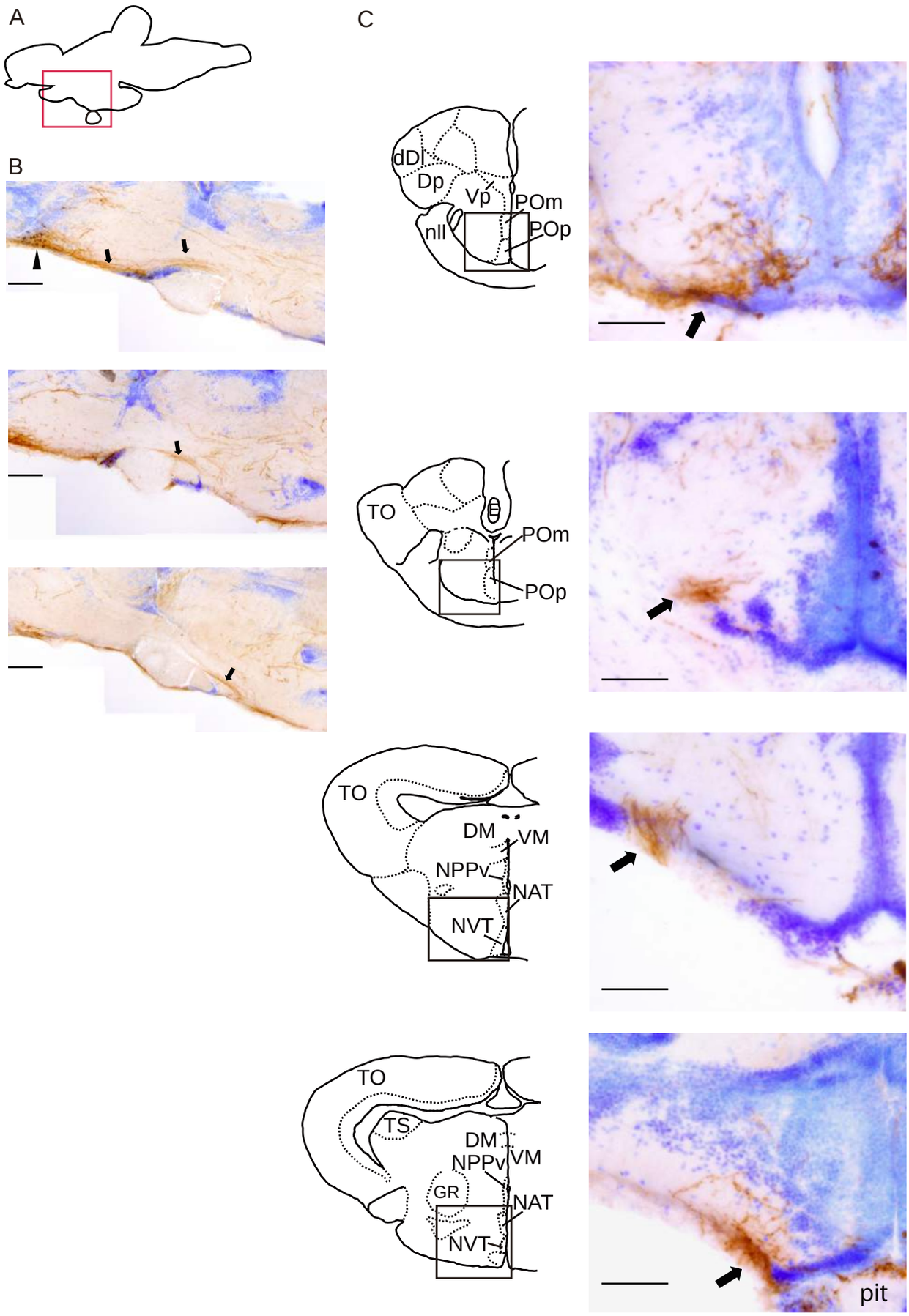

EGFP
erα mRNA
merge

Fig. 2


A
B
C
dDl
Dp
Vp
POm
POp
nll
TO
POm
POp
TO
DM
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NAT
NVT
TO
TS
DM
VM
NPPv
GR
NAT
NVT
pit

Fig. 3

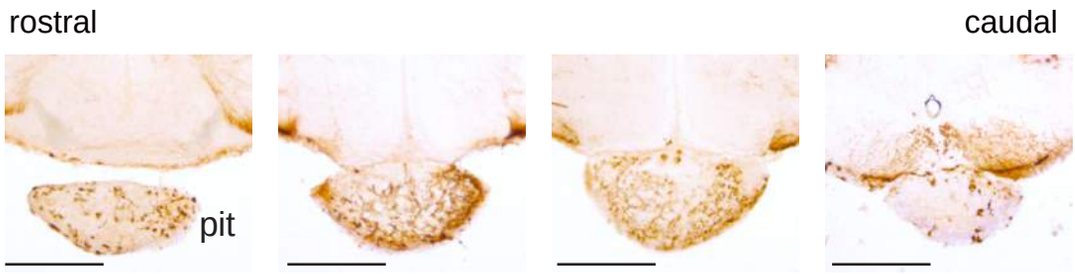
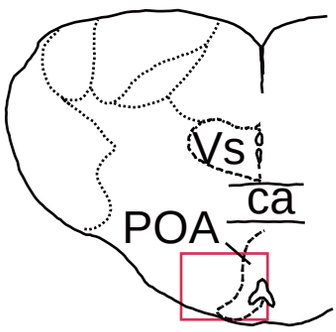


Fig. 4

A



B

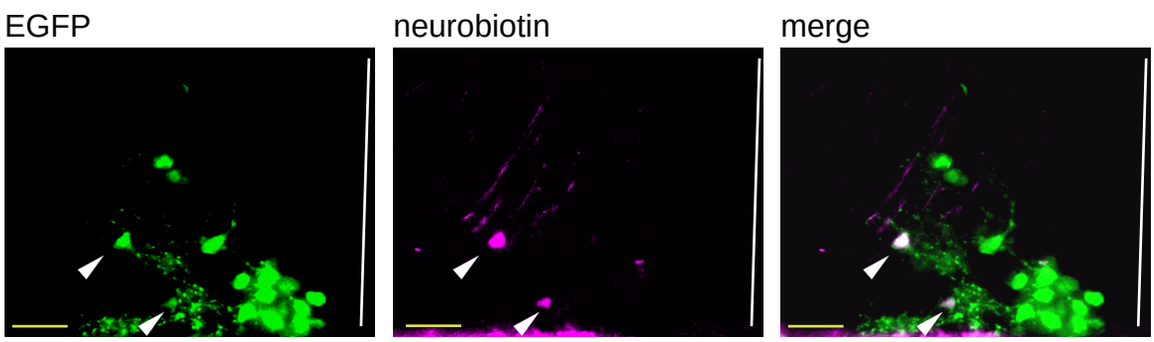


Fig. 5

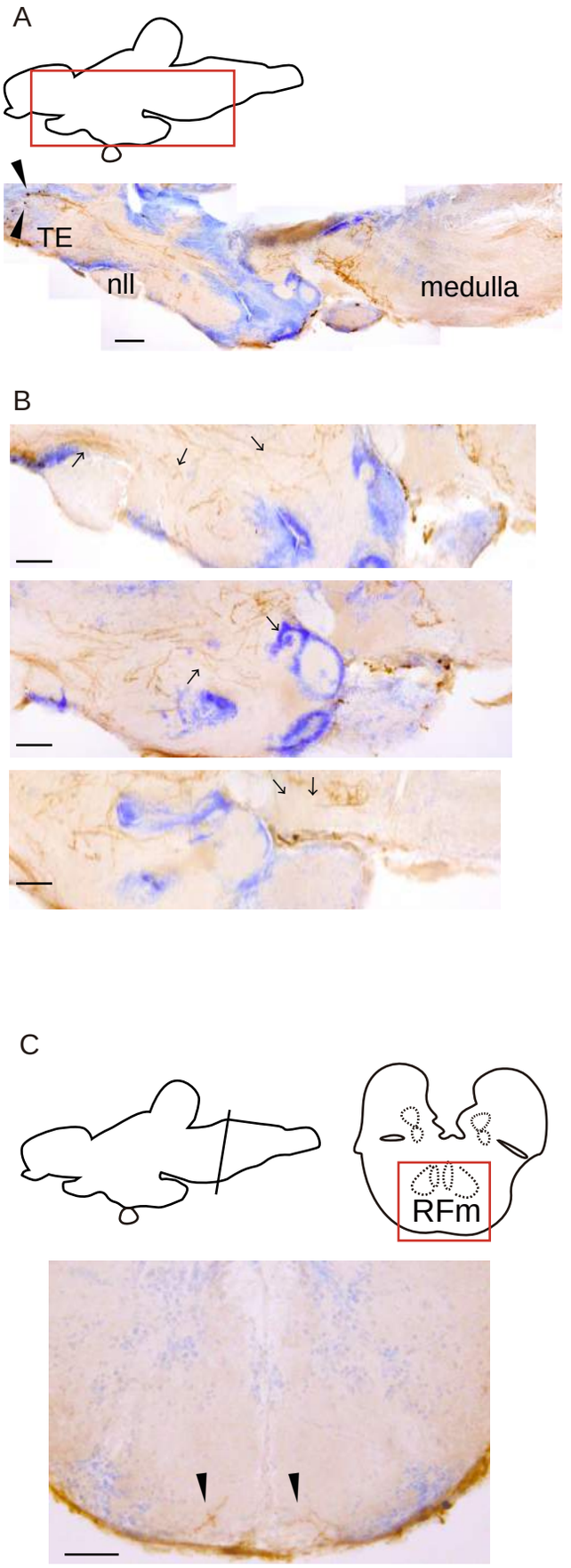


Fig. 6

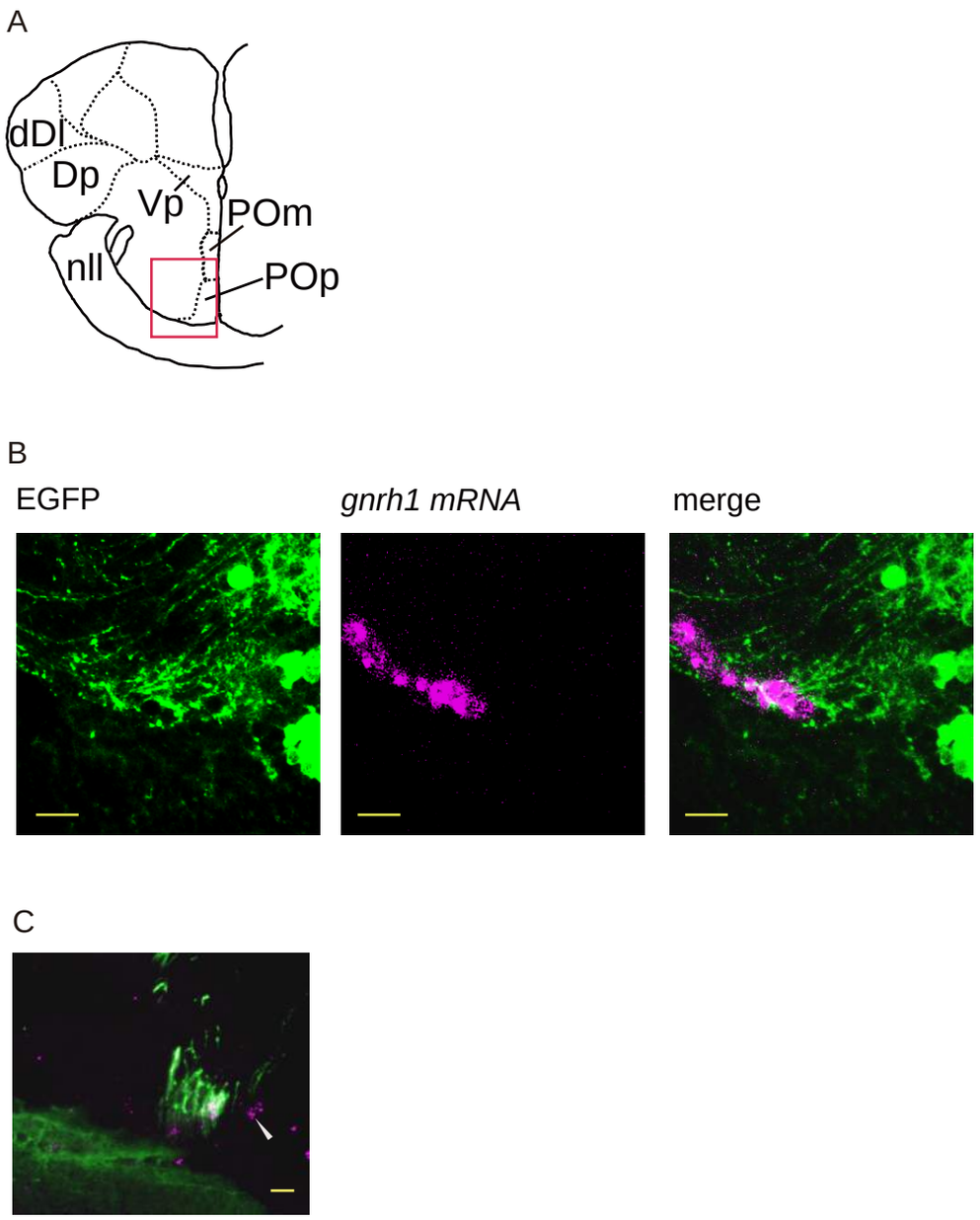
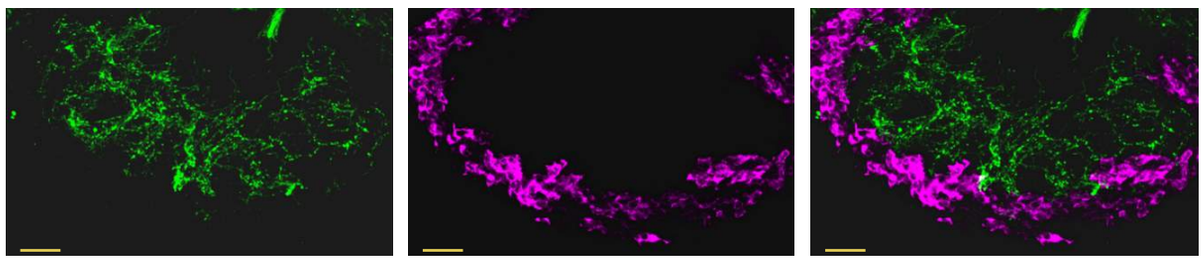


Fig. 7

A

EGFP *lhβ mRNA* merge



B

EGFP *fshβ mRNA* merge

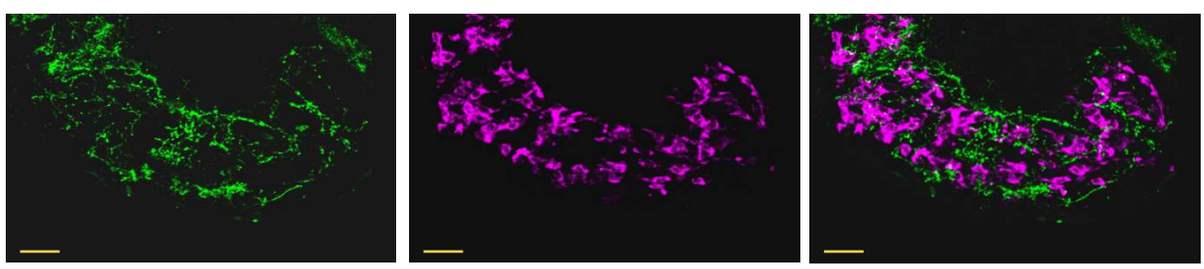


Fig. 8

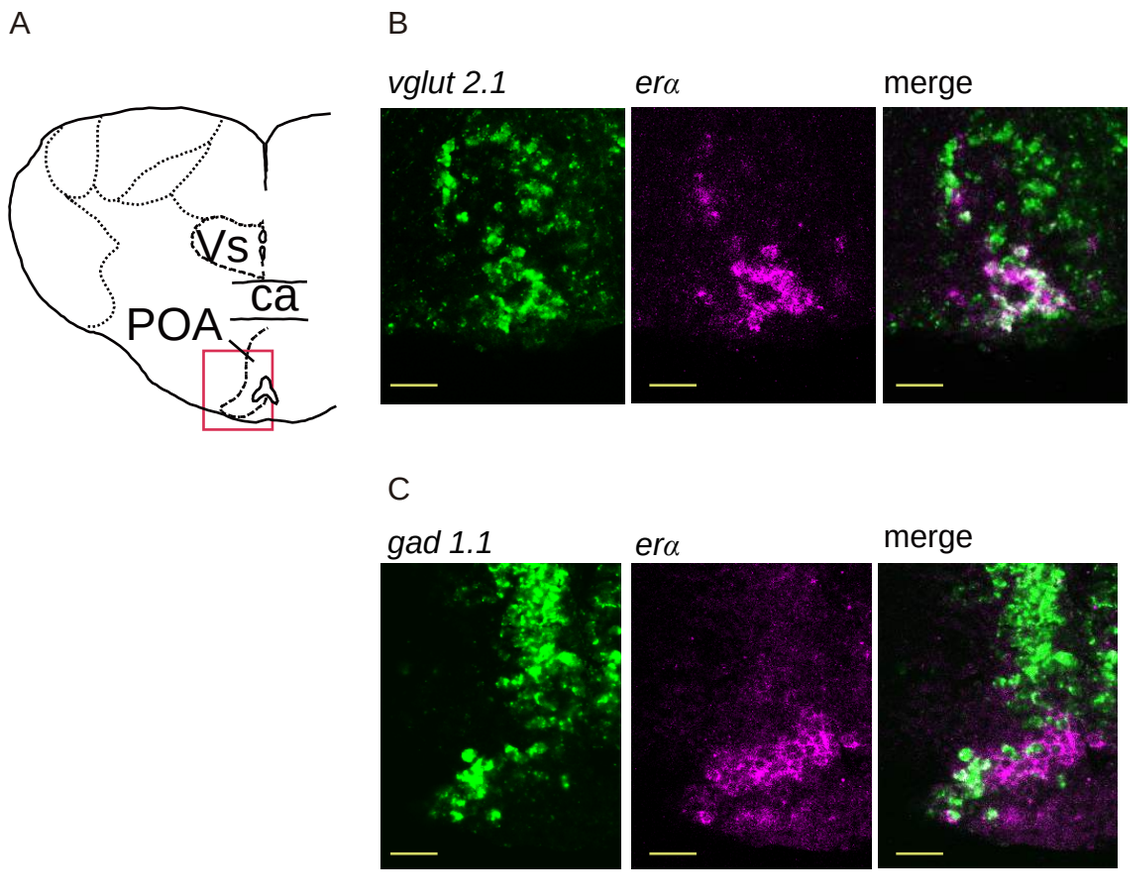
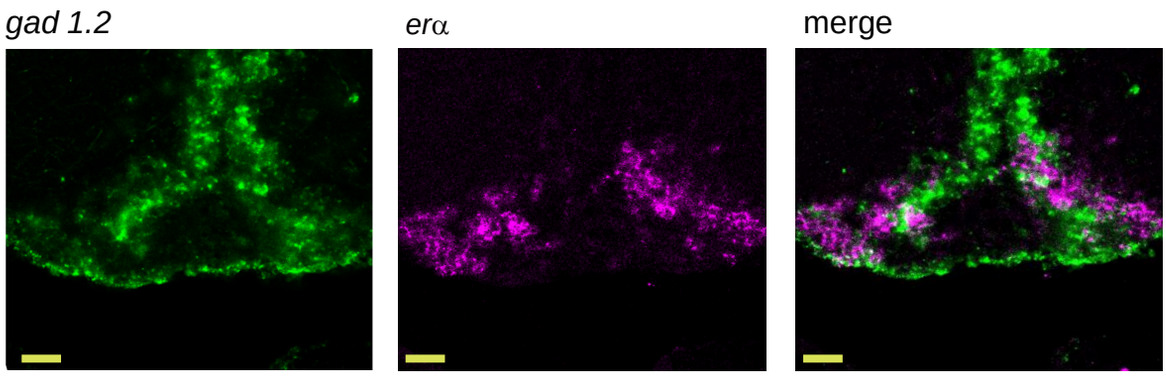
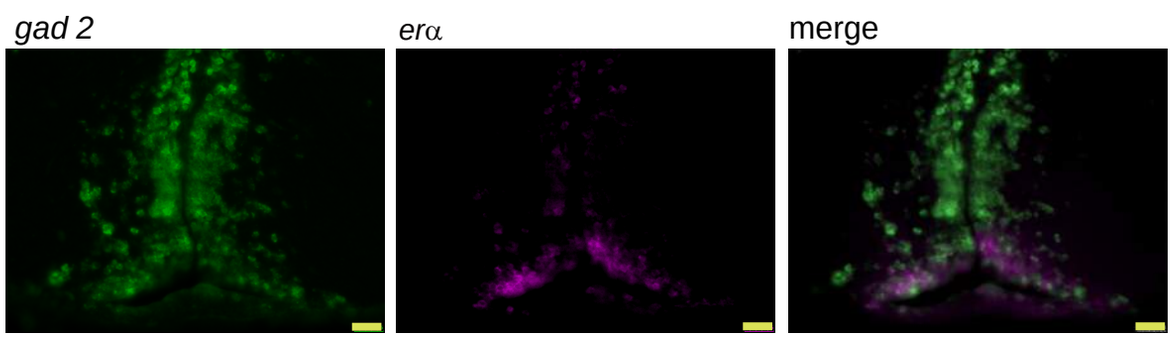


Fig. 9

A



B



General Discussion

The present thesis aims at clarifying the estrogenic regulation of reproduction in vertebrates using medaka as a model, with special reference to the ER. I detected target sites of estrogen from *in situ* hybridization analyses of ERs in the brain (Chapter1), and I also analyzed direct actions of estrogen on gonadotropes in the pituitary for the purpose of analyzing estrogenic regulation on the brain and the pituitary separately (Chapter2). In addition, I visualized ERα neurons by generating a transgenic (Tg) medaka line that expresses EGFP in the ERα neurons. By using this Tg medaka line, I analyzed neural circuitries that transmit gonadal estrogenic signals to the GnRH1 neurons and to the central pattern generator for sexual behaviors (Chapter3). In the present thesis, I suggested the importance of direct action of estrogen on the gonadotrope, and I also identified the glutamatergic/GABAergic neurons that relay estrogenic signals to the GnRH1 neurons for the first time in vertebrates.

Estrogen-primed neural circuits for the central regulation of sexual behaviors

In the present thesis, I suggested that all subtypes of ERs and ARs were distributed in Vs. Although previous studies suggested that neurons in Vs region are involved in the regulation of sexual behaviors, projections or neurotransmitters of these neurons in Vs have not been clarified. Here, I analyzed the projections of Vs-ERα neurons in the brain of *erα*:EGFP Tg medaka and suggested that Vs-ERα neurons project to medulla. It is possible that Vs-ERα neurons stimulate neurons in medulla, and the signal is considered to be transmitted to the neurons that evoke sexual behaviors. Although the neural circuits of sexual behaviors have not been clarified completely, it is possible that gonadal estrogen acts on the Vs-ERα neurons and regulate expression of neurotransmitters or firing activity, in accordance with the gonadal states. In this

scenario, Vs-ERα neurons regulate sexual behaviors in accordance with the circulating estrogen levels and enable animals to display sexual behaviors actively during the breeding season or after the sexual maturation.

Estrogen-primed circuits for the regulation of the HPG axis

In the present thesis, I proposed mechanisms for the estrogen feedback regulation of the HPG axis. In the present thesis, it was suggested that the estrogenic feedback signals regulate FSH through pathways different from those of LH. FSH expression was directly down-regulated by estrogen, while LH expression did not show significant change. Taken together with a previous study in the OVX medaka demonstrating that *in vivo* treatment of estrogen increases the expression of *lhβ* mRNA (Kanda et al., 2011), LH cells, compared with FSH, are considered to be more strongly regulated by inputs from the GnRH1 neurons. I also suggested that glutamatergic/GABAergic POA-ERα neurons are the regulators of GnRH1 neurons. In this scenario, the information on the gonadal status may be transmitted to the GnRH1 neurons by the glutamatergic/GABAergic POA-ERα neurons, and the GnRH1 neurons stimulate LH cells to induce ovulation in accordance with the elevation of circulating estrogen levels (Soyano et al., 1993). After spawning events following ovulation, the serum estrogen level decreases, and the transcription of *fshβ* is activated. By regulations via these different mechanisms, FSH and LH are released at different timings from each other and stimulate gonads to induce maturation of ovarian follicles or ovulation, respectively.

So far, many studies in mammals have suggested that glutamate and GABA regulate the activity of GnRH1 neurons in accordance with serum sex steroid concentrations (Christian and Moenter, 2007; Christian et al., 2009). However, in teleosts,

glutamate/GABA transmission to GnRH1 neurons has not been studied at all, because electrophysiological approach toward GnRH1 neurons has been limited to mice and rats in which transgenic animals are available. Moreover, it has been too difficult throughout vertebrates to anatomically analyze the GABAergic/glutamatergic neurons that regulate GnRH1 neurons, because there are so many glutamatergic/GABAergic neurons in the brain. In the present thesis, by visualizing ERα neurons with EGFP, I succeeded for the first time in enabling the specific identification of the glutamertagic/GABAergic neurons expressing ERα. The detailed anatomical analysis of this Tg medaka provided a novel information on the mechanisms of glutamate/GABA transmissions to the GnRH1 neurons. Taken together with the previous studies in mammals, it is possible that the actions of glutamate/GABA on GnRH1 neurons are common to both groups of animals and may be conserved throughout vertebrates. Further electrophysiological analysis of estrogen dependency of excitatory or inhibitory synaptic inputs from the POA-ERα positive glutamatergic/GABAergic neurons to the GnRH1 neurons in medaka should provide us with novel insights into the feedback regulation mechanism of the HPG axis of vertebrates in general.

It should be noted, however, that the present study does not rule out a possibility that the POA-ERα neurons release other neuropeptide(s), besides the classical transmitters glutamate and GABA, onto the GnRH1 neurons. Further analysis using my Tg medaka is a promising approach to examine this possibility. Anyway, GnRH1 neurons are essential for the HPG axis regulation throughout vertebrates, and it is conceivable that animals have developed diverse regulatory systems for GnRH1 neurons in accordance with their different patterns of reproduction, and all of the regulatory events start from

the sex steroid sensitive neurons, which I succeeded in visualization in the present thesis study.

In conclusion, I demonstrated estrogenic regulatory pathways for the neural circuits of sexual behaviors and for the HPG axis, from the brain nucleus level to the single neuron level, by combining genetic tools and detailed anatomical analysis. Via these estrogenic regulatory pathways, it is suggested that information on the gonadal status is transmitted to the brain and the pituitary, which enables animals to regulate the reproductive functions and the sexual behaviors in a coordinated manner. I believe that the present thesis study opens up a novel approach towards further understanding of the cooperative regulatory mechanisms of the reproductive functions in the neuroendocrine system.

References

Abe H, Oka Y. 2009. Primary culture of the isolated terminal nerve - GnRH neurones derived from adult teleost (dwarf gourami, *Colisa lalia*) brain for the study of peptide release mechanisms. Journal of Neuroendocrinology 21(5):489-505.

Aizen J, Kasuto H, Golan M, Zakay H, Levavi-Sivan B. 2007. Tilapia follicle-stimulating hormone (FSH): immunochemistry, stimulation by gonadotropin-releasing hormone, and effect of biologically active recombinant FSH on steroid secretion. Biology of Reproduction 76(4):692-700.

Akazome Y, Kanda S, Okubo K, Oka Y. 2010. Functional and evolutionary insights into vertebrate kisspeptin systems from studies of fish brain. Journal of Fish Biology 76(1):161-182.

Akazome Y, Kanda S, Oka Y. 2011. Expression of vesicular glutamate transporter-2.1 in medaka terminal nerve gonadotrophin-releasing hormone neurones. Journal of Neuroendocrinology 23(7):570-576.

Alves SE, Lopez V, McEwen BS, Weiland NG. 1998. Differential colocalization of estrogen receptor beta (ERbeta) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: an immunocytochemical study. Proceedings of the National Academy of Sciences of the United States of America 95(6):3281-3286.

Amano M, Oka Y, Aida K, Okumoto N, Kawashima S, Hasegawa Y. 1991. Immunocytochemical demonstration of salmon GnRH and chicken GnRH-II in the brain of masu salmon, *Oncorhynchus masou*. The Journal of Comparative Neurology 314(3):587-597.

Antonopoulou E, Bornestaf C, Swanson P, Borg B. 1999a. Feedback control of gonadotropins in Atlantic salmon, *Salmo salar*, *male parr.I.* Castration effects in rematuring and nonrematuring fish. General and Comparative Endocrinology 114(1):132-141.

Antonopoulou E, Mayer I, Borg B, Swanson P, Murza I, Christoforov O. 1999b. Effects of testosterone on gonadotropins, testes, and plasma 17alpha,20beta-dihydroxy-4-pregnene-3-one levels in postbreeding mature Atlantic salmon, *Salmo salar*, *male parr*. The Journal of Experimental Zoology 284(4):425-436.

Baird DT. 1983. Factors regulating the growth of the preovulatory follicle in the sheep and human. Journal of Reproduction and Fertility 69(1):343-352.

Bass AH. 2008. Steroid-dependent plasticity of vocal motor systems: novel insights from teleost fish. Brain Resesrch Review 57(2):299-308.

Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fischer WH, Vale WW. 2004. Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin. Molecular and Cellular Endocrinology 225(1-2):29-36.

Breton B, Govoroun M, Mikolajczyk T. 1998. GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: relationship with pituitary responsiveness to GnRH-A stimulation. General and Comparative Endocrinology 111(1):38-50.

Breton B, Sambroni E, Govoroun M, Weil C. 1997. Effects of steroids on GTH I and GTH II secretion and pituitary concentration in the immature rainbow trout *Oncorhynchus mykiss*. Comptes Rendus de l'Academie des Sciences Serie III, Sciences de la vie 320(10):783-789.

Calman B, Lin YW, Wallace RA. 2001. Preparation and use of specific antibodies to the beta-I and beta-II subunits of gonadotropic hormone from Fundulus heteroclitus pituitary. General and Comparative Endocrinology 123(2):203-209.

Cattanach BM, Iddon CA, Charlton HM, Chiappa SA, Fink G. 1977. Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. Nature 269(5626):338-340.

Childs. 2006. Gonadotropes and Lactotropes. Physiology of Reproduction 3rd ed. pp. 1483–1579.

Christian CA, Moenter SM. 2007. Estradiol induces diurnal shifts in GABA transmission to gonadotropin-releasing hormone neurons to provide a neural signal for ovulation. The Journal of Neuroscience : The Official Journal of The Society for Neuroscience 27(8):1913-1921.

Christian CA, Pielecka-Fortuna J, Moenter SM. 2009. Estradiol suppresses glutamatergic transmission to gonadotropin-releasing hormone neurons in a model of negative feedback in mice. Biology of Reproduction 80(6):1128-1135.

Christian CA, Moenter SM. 2010. The neurobiology of preovulatory and estradiol-induced gonadotropin-releasing hormone surges. Endocrine Reviews 31(4):544-577.

Couse JF, Yates MM, Walker VR, Korach KS. 2003. Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERalpha but not ERbeta. Molecular Endocrinology 17(6):1039-1053.

D'Aniello B, Pinelli C, Di Fiore MM, Tela L, King JA, Rastogi RK. 1995. Development and distribution of gonadotropin-releasing hormone neuronal systems in the frog (*Rana esculenta*) brain: immunohistochemical analysis. Developmental Brain Research 89(2):281-288.

de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54.

Proceedings of the National Academy of Sciences of the United States of America 100(19):10972-10976.

Dickey JT, Swanson P. 1998. Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). Journal of Molecular Endocrinology 21(3):291-306.

Dorling AA, Todman MG, Korach KS, Herbison AE. 2003. Critical Role for Estrogen Receptor alpha in Negative Feedback Regulation of Gonadotropin-Releasing Hormone mRNA Expression in the Female Mouse. Neuroendocrinology 78(4):204-209.

Escriva H, Delaunay F, Laudet V. 2000. Ligand binding and nuclear receptor evolution. BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology 22(8):717-727.

Fine ML, Chen FA, Keefer DA. 1996. Autoradiographic localization of dihydrotestosterone and testosterone concentrating neurons in the brain of the oyster toadfish. Brain research 709(1):65-80.

Forlano PM, Deitcher DL, Bass AH. 2005. Distribution of estrogen receptor alpha mRNA in the brain and inner ear of a vocal fish with comparisons to sites of aromatase expression. The Journal of Comparative Neurology 483(1):91-113.

Forlano PM, Marchaterre M, Deitcher DL, Bass AH. 2010. Distribution of androgen receptor mRNA expression in vocal, auditory, and neuroendocrine circuits in a teleost fish. The Journal of Comparative Neurology 518(4):493-512.

Galea LA, Uban KA, Epp JR, Brummelte S, Barha CK, Wilson WL, Lieblich SE, Pawluski JL. 2008. Endocrine regulation of cognition and neuroplasticity: our pursuit to unveil the complex interaction between hormones, the brain, and behaviour. Canadian Journal of Experimental Psychology = Revue Canadienne de Psychologie Experimentale 62(4):247-260.

Gelinas D, Callard GV. 1997. Immunolocalization of aromatase- and androgen receptor-positive neurons in the goldfish brain. General and Comparative Endocrinology 106(2):155-168.

Gieske MC, Kim HJ, Legan SJ, Koo Y, Krust A, Chambon P, Ko C. 2008. Pituitary gonadotroph estrogen receptor-alpha is necessary for fertility in females. Endocrinology 149(1):20-27.

Golan M, Biran J, Levavi-Sivan B. 2014. A novel model for development, organization, and function of gonadotropes in fish pituitary. Frontiers in Endocrinology 5:182.

Gorelick DA, Watson W, Halpern ME. 2008. Androgen receptor gene expression in the developing and adult zebrafish brain. Developmental Dynamics : An Official Publication of the American Association of Anatomists 237(10):2987-2995.

Greenwood AK, Wark AR, Fernald RD, Hofmann HA. 2008. Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an

African cichlid fish. Proceedings Biological Sciences / The Royal Society 275(1649):2393-2402.

Gregg DW, Nett TM. 1989. Direct effects of estradiol-17 beta on the number of gonadotropin-releasing hormone receptors in the ovine pituitary. Biology of Reproduction 40(2):288-293.

Guo XZ, Su JD, Sun QW, Jiao BH. 2001. Expression of estrogen receptor (ER) -alpha and -beta transcripts in the neonatal and adult rat cerebral cortex, cerebellum, and olfactory bulb. Cell Research 11(4):321-324.

Hamernik DL, Clay CM, Turzillo A, Van Kirk EA, Moss GE. 1995. Estradiol increases amounts of messenger ribonucleic acid for gonadotropin-releasing hormone receptors in sheep. Biology of Reproduction 53(1):179-185.

Hansel W, Convey EM. 1983. Physiology of the estrous cycle. Journal of Animal Science 57 Suppl 2:404-424.

Harbott LK, Burmeister SS, White RB, Vagell M, Fernald RD. 2007. Androgen receptors in a cichlid fish, *Astatotilapia burtoni*: structure, localization, and expression levels. The Journal of Comparative Neurology 504(1):57-73.

Hawkins MB, Godwin J, Crews D, Thomas P. 2005. The distributions of the duplicate oestrogen receptors ER-beta a and ER-beta b in the forebrain of the Atlantic croaker (*Micropogonias undulatus*): evidence for subfunctionalization after gene duplication. Proceedings Biological sciences / The Royal Society 272(1563):633-641.

Herbison AE, Moenter SM. 2011. Depolarising and hyperpolarising actions of GABA(A) receptor activation on gonadotrophin-releasing hormone neurones: towards an emerging consensus. Journal of Neuroendocrinology 23(7):557-569.

Hiraki T, Nakasone K, Hosono K, Kawabata Y, Nagahama Y, Okubo K. 2014. Neuropeptide B is female-specifically expressed in the telencephalic and preoptic nuclei of the medaka brain. Endocrinology 155(3):1021-1032.

Hiraki T, Takeuchi A, Tsumaki T, Zempo B, Kanda S, Oka Y, Nagahama Y, Okubo K. 2012. Female-specific target sites for both oestrogen and androgen in the teleost brain. Proceedings Biological sciences / The Royal Society 279(1749):5014-5023.

Hrabovszky E, Shughrue PJ, Merchenthaler I, Hajszan T, Carpenter CD, Liposits Z, Petersen SL. 2000. Detection of estrogen receptor-beta messenger ribonucleic acid and 125I-estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 141(9):3506-3509.

Huggard-Nelson DL, Nathwani PS, Kermouni A, Habibi HR. 2002. Molecular characterization of LH-beta and FSH-beta subunits and their regulation by estrogen in the goldfish pituitary. Molecular and Cellular Endocrinology 188(1-2):171-193.

Hull EM, Dominguez JM. 2007. Sexual behavior in male rodents. Hormones and Behavior 52(1):45-55.

Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA. 2004. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80(4):264-272.

Kanda S, Akazome Y, Matsunaga T, Yamamoto N, Yamada S, Tsukamura H, Maeda K, Oka Y. 2008. Identification of KiSS-1 product kisspeptin and steroid-sensitive sexually dimorphic kisspeptin neurons in medaka (*Oryzias latipes*). Endocrinology 149(5):2467-2476.

Kanda S, Akazome Y, Mitani Y, Okubo K, Oka Y. 2013. Neuroanatomical evidence that kisspeptin directly regulates isotocin and vasotocin neurons. PloS One 8(4):e62776.

Kanda S, Karigo T, Oka Y. 2012. Steroid sensitive kiss2 neurones in the goldfish: evolutionary insights into the duplicate kisspeptin gene-expressing neurones. Journal of Neuroendocrinology 24(6):897-906.

Kanda S, Okubo K, Oka Y. 2011. Differential regulation of the luteinizing hormone genes in teleosts and tetrapods due to their distinct genomic environments--insights into gonadotropin beta subunit evolution. General and Comparative Endocrinology 173(2):253-258.

Karigo T, Aikawa M, Kondo C, Abe H, Kanda S, Oka Y. 2014. Whole brain-pituitary in vitro preparation of the transgenic medaka (*Oryzias latipes*) as a tool for analyzing the differential regulatory mechanisms of LH and FSH release. Endocrinology 155(2):536-547.

Karigo T, Kanda S, Takahashi A, Abe H, Okubo K, Oka Y. 2012. Time-of-day-dependent changes in GnRH1 neuronal activities and gonadotropin mRNA expression in a daily spawning fish, medaka. Endocrinology 153(7):3394-3404.

Karigo T, Oka Y. 2013. Neurobiological study of fish brains gives insights into the nature of gonadotropin-releasing hormone 1-3 neurons. Frontiers in Endocrinology 4:177.

Kawai T, Abe H, Akazome Y, Oka Y. 2010. Neuromodulatory effect of GnRH on the synaptic transmission of the olfactory bulbar neural circuit in goldfish, *Carassius auratus*. Journal of Neurophysiology 104(6):3540-3550.

Kawai T, Oka Y, Eisthen H. 2009. The role of the terminal nerve and GnRH in olfactory system neuromodulation. Zoological Science 26(10):669-680.

Kim KH, Patel L, Tobet SA, King JC, Rubin BS, Stopa EG. 1999. Gonadotropin-releasing hormone immunoreactivity in the adult and fetal human olfactory system. Brain Research 826(2):220-229.

Kim Y, Ko E, Lappan S, Kezele A, Kwon SW, Choe JC. 2013. Changes in fecal estrogen levels and sexual behavior in captive sichuan snub-nosed monkeys (*Rhinopithecus roxellana*) following a male replacement. Zoo Biology 32(3):342-346.

Kim YS, Stumpf WE, Sar M. 1978. Topography of estrogen target cells in the forebrain of goldfish, *Carassius auratus*. The Journal of Comparative Neurology 182(4):611-620.

Kindler PM, Bahr JM, Philipp DP. 1991. The effects of exogenous 11-ketotestosterone, testosterone, and cyproterone acetate on prespawning and parental care behaviors of male bluegill. Hormones and Behavior 25(3):410-423.

Kinoshita M, Ito E. 2006. Roles of periventricular neurons in retinotectal transmission in the optic tectum. Progress in Neurobiology 79(2):112-121.

Kinoshita M, Ito E, Urano A, Ito H, Yamamoto N. 2006. Periventricular efferent neurons in the optic tectum of rainbow trout. The Journal of Comparative Neurology 499(4):546-564.

Kitamura S, Ogata H, Takashima F. 1994. Olfactory responses of several species of teleost to F-prostaglandins. Comparative Biochemistry and Physiology Comparative Physiology 107(3):463-467.

Kobayashi M, Aida K, Hanyu I. 1988. Hormone changes during the ovulatory cycle in goldfish. General and Comparative Endocrinology 69(2):301-307.

Koyama Y, Satou M, Oka Y, Ueda K. 1984. Involvement of the telencephalic hemispheres and the preoptic area in sexual behavior of the male goldfish, *Carassius auratus*: a brain-lesion study. Behavioral and Neural Biology 40(1):70-86.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Sciences of the United States of America 93(12):5925-5930.

Laflamme N, Nappi RE, Drolet G, Labrie C, Rivest S. 1998. Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. Journal of Neurobiology 36(3):357-378.

Larsen DA, Swanson P. 1997. Effects of gonadectomy on plasma gonadotropins I and II in coho salmon, *Oncorhynchus kisutch*. General and Comparative Endocrinology 108(1):152-160.

Larson ET, O'Malley DM, Melloni RH, Jr. 2006. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. Behavioural Brain Research 167(1):94-102.

Levavi-Sivan B, Biran J, Fireman E. 2006. Sex steroids are involved in the regulation of gonadotropin-releasing hormone and dopamine D2 receptors in female tilapia pituitary. Biology of Reproduction 75(4):642-650.

Liley NR. 1972. The effects of estrogens and other steroids on the sexual behavior of the female guppy, *Poecilia reficulata*. General and Comparative Endocrinology Supplyment 3: 542-552.

Lin SW, Ge W. 2009. Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish--an in vitro approach. General and Comparative Endocrinology 160(2):183-193.

Luine VN. 2008. Sex steroids and cognitive function. Journal of Neuroendocrinology 20(6):866-872.

Maruska KP, Fernald RD. 2010. Reproductive status regulates expression of sex steroid and GnRH receptors in the olfactory bulb. Behavioural Brain Research 213(2):208-217.

Mateos J, Mananos E, Carrillo M, Zanuy S. 2002. Regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotropin-releasing hormone (GnRH) and sexual steroids in the Mediterranean Sea bass. Comparative Biochemistry and Physiology Part B, Biochemistry & Molecular Biology 132(1):75-86.

McCormick CA, Hernandez DV. 1996. Connections of octaval and lateral line nuclei of the medulla in the goldfish, including the cytoarchitecture of the secondary octaval population in goldfish and catfish. Brain, Behavior and Evolution 47(3):113-137.

Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O, Pakdel F. 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biology of Reproduction 66(6):1881-1892.

Merchenthaler I, Lane MV, Numan S, Dellovade TL. 2004. Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses. The Journal of Comparative Neurology 473(2):270-291.

Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA. 2005. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proceedings of the National Academy of Sciences of the United States of America 102(5):1761-1766.

Miller WL, Alexander DC, Wu JC, Huang ES, Whitfield GK, Hall SH. 1983. Regulation of beta-chain mRNA of ovine follicle-stimulating hormone by 17 beta-estradiol. Molecular and Cellular Biochemistry 53-54(1-2):187-195.

Mitani Y, Kanda S, Akazome Y, Zempo B, Oka Y. 2010. Hypothalamic Kiss1 but not Kiss2 neurons are involved in estrogen feedback in medaka (*Oryzias latipes*). Endocrinology 151(4):1751-1759.

Morali G, Larsson K, Beyer C. 1977. Inhibition of testosterone-induced sexual behavior in the castrated male rat by aromatase blockers. Hormones and Behavior 9(3):203-213.

Munakata A, Kobayashi M. 2010. Endocrine control of sexual behavior in teleost fish. General and Comparative Endocrinology 165(3):456-468.

Munchrath LA, Hofmann HA. 2010. Distribution of sex steroid hormone receptors in the brain of an African cichlid fish, *Astatotilapia burtoni*. The Journal of Comparative Neurology 518(16):3302-3326.

Muriach B, Carrillo M, Zanuy S, Cerda-Reverter JM. 2008. Distribution of estrogen receptor 2 mRNAs (Esr2a and Esr2b) in the brain and pituitary of the sea bass (*Dicentrarchus labrax*). Brain Research 1210:126-141.

Murr SM, Geschwind, II, Bradford GE. 1973. Plasma LH and FSH during different oestrous cycle conditions in mice. Journal of Reproduction and Fertility 32(2):221-230.

Nett TM, Turzillo AM, Baratta M, Rispoli LA. 2002. Pituitary effects of steroid hormones on secretion of follicle-stimulating hormone and luteinizing hormone. Domestic animal Endocrinology 23(1-2):33-42.

Ogino Y, Katoh H, Kuraku S, Yamada G. 2009. Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates. Endocrinology 150(12):5415-5427.

Ohya T, Hayashi S. 2006. Vasotocin/isotocin-immunoreactive neurons in the medaka fish brain are sexually dimorphic and their numbers decrease after spawning in the female. Zoological Science 23(1):23-29.

Oka Y. 2009. Three types of gonadotrophin-releasing hormone neurones and steroid-sensitive sexually dimorphic kisspeptin neurones in teleosts. Journal of Neuroendocrinology 21(4):334-338.

Oka Y. 2010. Electrophysiological characteristics of gonadotrophin-releasing hormone 1-3 neurones: insights from a study of fish brains. Journal of Neuroendocrinology 22(7):659-663.

Oka Y, Matsushima T. 1993. Gonadotropin-releasing hormone (GnRH)-immunoreactive terminal nerve cells have intrinsic rhythmicity and project widely in the brain. The Journal of Neuroscience : The Official Journal of The Society for Neuroscience 13(5):2161-2176.

Okubo K, Nagahama Y. 2008. Structural and functional evolution of gonadotropin-releasing hormone in vertebrates. Acta Physiologica 193(1):3-15.

Okubo K, Sakai F, Lau EL, Yoshizaki G, Takeuchi Y, Naruse K, Aida K, Nagahama Y. 2006. Forebrain gonadotropin-releasing hormone neuronal development: insights from transgenic medaka and the relevance to X-linked Kallmann syndrome. Endocrinology 147(3):1076-1084.

Pfaff DW, Pfaffmann C. 1969. Olfactory and hormonal influences on the basal forebrain of the male rat. Brain Research 15(1):137-156.

Pfaff DW, Sakuma Y. 1979. Facilitation of the lordosis reflex of female rats from the ventromedial nucleus of the hypothalamus. The Journal of Physiology 288:189-202.

Pielecka-Fortuna J, Chu Z, Moenter SM. 2008. Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. Endocrinology 149(4):1979-1986.

Portillo W, Diaz NF, Cabrera EA, Fernandez-Guasti A, Paredes RG. 2006. Comparative analysis of immunoreactive cells for androgen receptors and oestrogen receptor alpha in copulating and non-copulating male rats. Journal of Neuroendocrinology 18(3):168-176.

Prat F, Sumpter JP, Tyler CR. 1996. Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductivecycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biology of Reproduction 54(6):1375-1382.

Salek SJ, Sullivan CV, Godwin J. 2002. Arginine vasotocin effects on courtship behavior in male white perch (*Morone americana*). Behavioural Brain Research 133(2):177-183.

Santangelo N, Bass AH. 2006. New insights into neuropeptide modulation of aggression: field studies of arginine vasotocin in a territorial tropical damselfish. Proceedings Biological Sciences / The Royal Society 273(1605):3085-3092.

Satou M, Oka Y, Kusunoki M, Matsushima T, Kato M, Fujita I, Ueda K. 1984. Telencephalic and preoptic areas integrate sexual behavior in hime salmon (landlocked red salmon, *Oncorhynchus nerka*): results of electrical brain stimulation experiments. Physiology & Behavior 33(3):441-447.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH. 2003. The GPR54 gene as a regulator of puberty. The New England Journal of Medicine 349(17):1614-1627.

Semsar K, Kandel FL, Godwin J. 2001. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. Hormones and Behavior 40(1):21-31.

Shapiro RA, Xu C, Dorsa DM. 2000. Differential transcriptional regulation of rat vasopressin gene expression by estrogen receptor alpha and beta. Endocrinology 141(11):4056-4064.

Shughrue PJ, Lane MV, Merchenthaler I. 1997. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. The Journal of Comparative Neurology 388(4):507-525.

Shupnik MA. 1996. Gonadal hormone feedback on pituitary gonadotropin genes. Trends in Endocrinology and Metabolism: TEM 7(8):272-276.

Simerly RB, Chang C, Muramatsu M, Swanson LW. 1990. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. The Journal of Comparative Neurology 294(1):76-95.

Singh SP, Wolfe A, Ng Y, DiVall SA, Buggs C, Levine JE, Wondisford FE, Radovick S. 2009. Impaired estrogen feedback and infertility in female mice with pituitary-specific deletion of estrogen receptor alpha (ESR1). Biology of Reproduction 81(3):488-496.

Singh U, Kumar S, Singru PS. 2012. Interaction between dopamine- and isotocin-containing neurones in the preoptic area of the catfish, *Clarias batrachus*: role in the regulation of luteinising hormone cells. Journal of Neuroendocrinology 24(11):1398-1411.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. 2005. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146(9):3686-3692.

Smith RJ. 1969. Control of prespawning behaviour of sunfish (*Lepomis gibbosus* and *L. megalotis*). I. Gonadal androgen. Animal Behaviour 17(2):279-285.

Soyano K, Saito T, Nagae M, Yamauchi K. 1993. Effects of thyroid hormone on gonadotropin-induced steroid production in medaka, *Oryzias latipes*, ovarian follicles. Fish Physiology and Biochemistry 11(1-6):265-272.

Stacey N, Kobayashi M. 1996. Androgen induction of male sexual behaviors in female goldfish. Hormones and Behavior 30(4):434-445.

Stachenfeld NS, Taylor HS. 2014. Challenges and methodology for testing young healthy women in physiological studies. American Journal of Physiology Endocrinology and Metabolism 306(8):E849-853.

Suzuki K, Kawauchi H, Nagahama Y. 1988. Isolation and characterization of subunits from two distinct salmon gonadotropins. General and Comparative Endocrinology 71(2):302-306.

Tang H, Liu Y, Luo D, Ogawa S, Yin Y, Li S, Zhang Y, Hu W, Parhar IS, Lin H, Liu X, Cheng CH. 2014. The kiss/kissr systems are dispensable for zebrafish reproduction: evidence from gene knockout studies. Endocrinology:en20141204.

Terasawa E, Fernandez DL. 2001. Neurobiological mechanisms of the onset of puberty in primates. Endocrine Reviews 22(1):111-151.

Teruyama R, Beck MM. 2000. Changes in immunoreactivity to anti-cGnRH-I and -II are associated with photostimulated sexual status in male quail. Cell and Tissue Research 300(3):413-426.

Trudeau VL, Murthy CK, Habibi HR, Sloley BD, Peter RE. 1993. Effects of sex steroid treatments on gonadotropin-releasing hormone-stimulated gonadotropin secretion from the goldfish pituitary. Biology of Reproduction 48(2):300-307.

Vadakkadath Meethal S, Atwood CS. 2005. The role of hypothalamic-pituitary-gonadal hormones in the normal structure and functioning of the brain. Cellular and Molecular Life Sciences : CMLS 62(3):257-270.

Van den Dungen HM, Buijs RM, Pool CW, Terlou M. 1982. The distribution of vasotocin and isotocin in the brain of the rainbow trout. The Journal of Comparative Neurology 212(2):146-157.

Vanderhorst VG, Gustafsson JA, Ulfhake B. 2005. Estrogen receptor-alpha and -beta immunoreactive neurons in the brainstem and spinal cord of male and female mice: relationships to monoaminergic, cholinergic, and spinal projection systems. The Journal of Comparative Neurology 488(2):152-179.

Wintermantel TM, Campbell RE, Porteous R, Bock D, Grone HJ, Todman MG, Korach KS, Greiner E, Perez CA, Schutz G, Herbison AE. 2006. Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. Neuron 52(2):271-280.

Wirsig-Wiechmann CR, Lee CE. 1999. Estrogen regulates gonadotropin-releasing hormone in the nervus terminalis of *Xenopus laevis*. General and Comparative Endocrinology 115(2):301-308.

Witkin JW. 1996. Effects of ovariectomy on GnRH neuronal morphology in rhesus monkey (*Macaca mulatta*). Journal of Neuroendocrinology 8(8):601-604.

Witkin JW, Ferin M, Popilskis SJ, Silverman AJ. 1991. Effects of gonadal steroids on the ultrastructure of GnRH neurons in the rhesus monkey: synaptic input and glial apposition. Endocrinology 129(2):1083-1092.

Yamamoto N, Kato T, Okada Y, Somiya H. 2010. Somatosensory nucleus in the torus semicircularis of cyprinid teleosts. The Journal of Comparative Neurology 518(13):2475-2502.

Yamamoto N, Oka Y, Kawashima S. 1997. Lesions of gonadotropin-releasing hormone-immunoreactive terminal nerve cells: effects on the reproductive behavior of male dwarf gouramis. Neuroendocrinology 65(6):403-412.

Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B. 2003. Regulation of fish gonadotropins. International Review of Cytology 225:131-185.

Yen FP, Lee YH, He CL, Huang JD, Sun LT, Dufour S, Chang CF. 2002. Estradiol-17beta triggers luteinizing hormone release in the protandrous black porgy (*Acanthopagrus schlegeli Bleeker*) through multiple interactions with gonadotropin-releasing hormone control. Biology of Reproduction 66(1):251-257.

Yuen CW, Ge W. 2004. Follistatin suppresses FSHbeta but increases LHbeta expression in the goldfish - evidence for an activin-mediated autocrine/paracrine system in fish pituitary. General and Comparative Endocrinology 135(1):108-115.

Zempo B, Kanda S, Okubo K, Akazome Y, Oka Y. 2013. Anatomical distribution of sex steroid hormone receptors in the brain of female medaka. The Journal of Comparative Neurology 521(8):1760-1780.

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