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Some hypothalamic neurons expressing estrogen receptor α (Esr1) are thought to transmit a gonadal estrogen feedback signal to gonadotropin releasing hormone 1 (GnRH1) neurons, which is the final common pathway for feedback regulation of reproductive functions. Moreover, estrogen-sensitive neurons are suggested to control sexual behaviors in coordination with reproduction. In mammals, hypothalamic estrogen-sensitive neurons release the peptide Kisspeptin and regulate GnRH1 neurons. However, a growing body of evidence in non-mammalian species casts doubt on the regulation of GnRH1 neurons by Kisspeptin neurons. As a first step towards understanding how estrogen regulates neuronal circuits for reproduction and sex behavior in vertebrates in general, we generated a transgenic medaka that expresses EGFP specifically in Esr1-expressing neurons (Esr1 neurons) and analyzed their axonal projections. We found that Esr1 neurons in the POA project to the GnRH1 neurons. We also demonstrated, by transcriptome and histological analyses, that these Esr1 neurons are glutamatergic and/or GABAergic, but not Kisspeptinergic. We therefore suggest that glutamatergic/GABAergic Esr1 neurons in the POA regulate GnRH1 neurons. This hypothesis is consistent with previous studies in mice that glutamatergic/GABAergic transmission is critical for estrogen-dependent changes in GnRH1 neuron firing. Thus, we propose that this neuronal circuit may provide an evolutionarily conserved mechanism for regulation of reproduction. In addition, we showed that telencephalic Esr1 neurons project to medulla, which may control sexual behavior. Moreover, we found that some POA-Esr1 neurons co-express progesterone receptors (PRs). These

neurons may form the neuronal circuits that regulate reproduction and sex behavior in response to the serum estrogen/progesterone.

Analyses of *esr1*:EGFP transgenic medaka brains showed that glutamatergic or GABAergic POA-Esr1/PR neurons project to GnRH1 neurons and to pituitary, and telencephalic Esr1 neurons project to medulla.

Introduction

Reproduction in vertebrates is regulated by the so-called hypothalamo-pituitary-gonadal (HPG) axis in a well-coordinated manner. Here, gonadotropin releasing hormone (GnRH), which is now often called GnRH1 for evolutionary reasons (1), acts on the pituitary to facilitate the release of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH, in turn, stimulate gonads and induce their maturation (2,3). The mature gonads release sex steroids into the circulation. Then, the sex steroids, especially estrogen, stimulate or inhibit the hypothalamus to up- or down-regulate release of GnRH1. This entire process is called “steroid feedback”. Recently, the importance of each component of HPG axis regulation in teleosts (GnRH1, LH, and FSH) has been scrutinized using gene knockout techniques in zebrafish and medaka (4,5), suggesting the importance and the evolutionary conservation of basic HPG axis regulation mechanisms in vertebrates, although there are some important differences between mammals and teleosts.

In HPG axis regulation, gonadal estrogen may be considered to be one of the most important signals that transmit the reproductive status of the gonad to the central nervous system, and its mechanisms of action have been analyzed intensively. It has been generally suggested that there are two types of estrogen receptors (Esrs), Esr1 (or ERα, coded by *esr1*) and Esr2 (or ERβ, coded by *esr2*); further, data from knockout mice suggest that Esr1 but not Esr2 is essential for reproduction (6,7).

A growing body of evidence in mammals suggests that Esr1 is expressed in Kisspeptin neurons rather than in GnRH1 neurons (8-10), and that Kisspeptin neurons regulate the firing activity of GnRH1 neurons and GnRH1 release in the median eminence in response to serum estrogen levels. Although the expression of Esr1 in Kisspeptin neurons has been reported to be widely conserved in vertebrates including teleosts (11,12), accumulating evidence argues against the presence of robust regulation of GnRH1 neurons by Kisspeptin neurons, and hence their role in regulation of reproduction in non-mammalian vertebrates. For example, GnRH1 neurons in teleosts do not express Kisspeptin receptors (neither GPR54 paralogs, *gpr54-1* nor *gpr54-2*) (13-15), and zebrafish with *kiss1* and *kiss2* double knockout show normal fertility (16). Furthermore, it is already known that avian species completely lack *kisspeptin* genes (17). Therefore, presumably unidentified estrogen-sensitive neurons other than Kisspeptin neurons play important roles in the HPG axis regulation in non-mammalian vertebrates.

On the other hand, it is also well known that sex steroids modulate specific behaviors depending on reproductive conditions. For example, most vertebrates show species-specific courtship behavior only after sexual maturation and during the breeding season. Thus, hormonal signals from the gonads have been suggested to control neural circuits for the modulation of sexual behaviors (18,19). However, the nature of such estrogen-sensitive neurons remains to be elucidated.

With these unanswered questions in mind, we aimed to anatomically identify the location and axonal projections of Esr1-expressing neurons mediating estrogen feedback signals to GnRH1 neurons, as well as those mediating sex steroid-sensitive signals to circuits that modulate sex behaviors. Here, we generated transgenic animals in which Esr1-expressing neurons are labeled with EGFP in the live brain for later detailed anatomical and physiological analyses. We chose medaka because of the following experimental advantages. First, molecular genetic tools can be applied rather easily. Second, female medaka spawn regularly every day under long-day conditions, and their breeding/nonbreeding states can be experimentally controlled by changing the day length. In addition, we have already described the anatomical distribution of *esr* mRNA expression in the medaka brain using *in situ* hybridization (20). In the present study, we further detailed the axonal projections of Esr1-expressing neurons (Esr1 neurons) using immunohistochemical methods to detect EGFP. Because Esrs are nuclear receptors, it is not possible to visualize projections of Esr1 neurons using antibody directed against the Esr peptide. Therefore, we generated a novel transgenic medaka line that expresses EGFP specifically in Esr1 neurons. In these animals, EGFP that is expressed under the regulation of the *esr1* promoter passively diffuses in the entire cytoplasm, enabling visualization of the entire neuronal morphology of Esr1 neurons including axons and dendrites.

Materials and methods

Animals

All the experiments including the generation of transgenic line were conducted using d-rR strain medaka (*Oryzias latipess*), a teleost fish. For each experiment, sexually mature, gonadally intact female wild type and/or transgenic animals were used. Medaka 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. All experiments were conducted in accordance with the protocols approved by the Animal Care and Use Committee of the University of Tokyo (permission number: 15–3).

Generation of transgenic medaka

We generated constructs for *esr1*: enhanced green fluorescein protein (EGFP) Tg medaka using a double-promoter approach for efficient screening. Using the zebrafish *cardiac myosin light chain 2* (*cmlc2*) promoter, we visualized the heart for screening transgene-positive

embryos. We carried out PCR using PrimeSTAR polymerase (Takara, Shiga, Japan) to amplify a medaka genomic DNA fragment containing the 5'-flanking region (3.7- kb) of exon 2 of the *esr1* gene from a bacterial artificial chromosome clone (BAC; clone number Golwb109_F22) from a medaka strain, HdrR, using a forward/reverse primer pair, P*esr1*-F (5'-ACAGGATGGAGGTCAAAAGC-3') /P*esr1*-R (GACCCCCTCGGTGACATGTATCCACCGGTCGCCACCATGG-3'). P*esr1*-F (5′-CAGAACTTCCTTGCTCATGCTCACC-3′)/P*esr1*-R LINKEGFPhind (5′-GAGAAGCTTCAGAGCCCTTCCCCTGTGCTCAGGC-3′). For gene IDs, see Table 2. Then, we fused this amplicon with an EGFP open reading frame by overlap extension PCR. Downstream of the coding sequence of EGFP, we fused the *cmlc2* promoter region and I-SceI restriction enzyme site. An approximately 6-kb fragment was then cloned into the TOPO-XL cloning vector (Invitrogen, Carlsbad, CA). For microinjection and screening, we followed a protocol that has been described previously (21). After generation of the transgenic line, we confirmed the specificity of EGFP expression in Esr1 neurons in brain sections by double labeling with *esr1 in situ* hybridization and EGFP immunohistochemistry. The brains of fish from the *esr1*:EGFP transgenic line were fixed with 4% PFA in PBS. The fixed brains were frontally cryo-sectioned at 20 μm using a cryostat (CM 3050S; Leica Microsystems) and mounted onto MAS-GP type A coated glass slides (Matsunami, Osaka, Japan). Sections were incubated with anti-EGFP antibody raised in rabbit (generous gift from Drs. Kaneko and Hioki, Kyoto University, Kyoto, Japan, see also Table1, antibody table) diluted 1:1000 with PBS containing 0.3% Tween 20 (PBST) overnight, rinsed twice with PBST, and incubated with biotinylated anti-rabbit IgG (Invitrogen) (diluted 1:200 with PBST) for 2 h. Then, sections were fixed by 4% PFA in PBS for 15 min, and rinsed with PBS containing 0.2% glycine. Next, we performed *in situ* hybridization using these slides to detect *esr1*, *esr2a* or *esr2b* mRNA using *esr1*-, *esr2a*-, or *esr2b*-specific DIG-labeled probes that were described in a previous report (11, 20) following a protocol described previously (20). Briefly, slides were incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Sections were then washed with PBS and prehybridized at 58°C for at least 30 min in a 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). 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. The 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. They 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 7.5; 0.16 M NaCl; and 0.1% Tween 20) for 5 min followed by 1.5% blocking reagent with DIG-1 for 30 min and DIG-1 for 15 min, and then 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. 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. Sections were then rinsed twice with DIG-1 buffer. After detection of EGFP signals, alkaline phosphatase activity, which was used to label mRNA, was detected using a Fast-Red substrate kit (Roche) according to the manufacturer's instructions. Incubation for this substrate was carried out until visible signals were detected and was stopped by washing in PBS containing 0.5 mM EDTA. Then, the sections were coverslipped with CC/Mount (Diagnostic BioSystems). Fluorescence was observed using a confocal laser-scanning microscope (LSM-710, Carl Zeiss, Oberkochen, Germany). We calculated the percentage of EGFP labeled *esr1* expressing cells by counting the number of both EGFP positive and negative *esr1* expressing cells. Numbers of cells were expressed as means ± SD.

RNA sequencing (RNA-seq)

Brains of sexually mature female *esr1*:EGFP transgenic medaka were dissected out under an upright fluorescent microscope (Eclipse E600FN; Nikon, Tokyo, Japan) using a method described in our previous study (22). Brains were kept in a chamber filled with ACSF, and a constant flow (1mL/min) of ACSF was applied 15 min prior to and during cell harvesting using a peristaltic pump to clean debris from dead/unhealthy cells. Patch pipettes of borosilicate glass capillaries with an outer diameter of 1.5-mm (GD-1.5; Narishige, Tokyo, Japan) were pulled to produce 3~5µm tip using a micropipette puller (P-97; Sutter Instruments). The tip of the capillary was back-filled by capillarity with a nuclease-free water (<1µL). Slight positive pressure was applied to patch pipettes while approaching the targeted EGFP-positive neurons and then EGFP-positive neurons were carefully pulled into the capillary by a negative pressure. The contents of the pipette were expelled into a 0.2 µL tube containing 10 µL Buffer RLT (Qiagen) with 1% 2-mercaptoethanol. Using this approach, five samples consisting of five neurons each were prepared from five different fish. Samples were frozen at -80°C immediately after harvesting. Total RNA was purified with Ampure XP RNA (Beckman Coulter). For reverse transcription, whole-transcript amplification and RNA-seq library preparation, we followed the Quartz-seq protocol (23). RNA-seq was performed with HiSeq1500 (Illumina) using the HiSeq Rapid SBS Kit v1 following manufacturer's instructions. For data analysis, we used the medaka genome (ftp://ftp.ensembl.org/pub/release-79/gtf/oryzias_latipes). At least 13 million sequences of 50-base single-reads were mapped onto the genome for each sample. Calculations of read mapping and reads per kilobase (kb)

of transcript per million mapped reads (RPKM) were performed using the CLCbio Genomics workbench software (CLC Bio, Aarhus, Denmark).

Double labeling for EGFP and mRNA of *gnrh1* or *pr*

We performed double labeling for EGFP and mRNA of *gnrh1* or *pr* as described above. To detect *pr* mRNA, we prepared a specific digoxigenin (DIG)-labeled riboprobe. Nested primers used to amplify medaka-*pr*-cDNAs were: first round, *pr*-F1 5'-ATGGAGAGTAAAATGAACGGAAAGCTGG-3' and *pr*-R1 5'-GTCATAGCCGGAGTACACGGTCT-3'; second round, *pr*-F2 5'-CCGAGTCCAGAGTAAATGGCTTGATCGA-3' and *pr*-R2 5'-CGGCTCGATGTTCTCCAGAATGT-3'. The template used to make the *pr* probe was 1.1-kb in length (DDBJ/EMBL/ GenBank accession no. AB360545.1). The probe for *pr* was synthesized from the medaka brain using a labeling kit (Roche Molecular Biochemicals GmbH, Mannheim, Germany). We performed *in situ* hybridization using a *gnrh1*-specific DIG-labeled probe that was generated previously (22). We calculated the percentage of *pr* expressing cells that were labeled with EGFP by counting the number of both EGFP positive and negative *pr* expressing cells. We also calculated the percentage of GnRH1 neurons that received projections from EGPF fibers by counting the number of cell bodies of GnRH1 neurons that were surrounded by EGFP labeled fibers within 1 μm. For both calculations, number of cells was expressed as mean ± SD.

Immunohistochemistry for tract tracing

We carried out immunohistochemistry for EGFP to analyze axonal projections of Esr1 neurons in detail. All the procedures were performed as described above up to the secondary antibody, which was a biotinylated anti-rabbit IgG. 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, the slides were washed twice with PBST and reacted with 3, 3'-diaminobenzidine. For some slides, we performed Nissl counter-staining using a 0.1% cresyl violet solution. The slides were then dehydrated, cleared, and coverslipped. Photographs were taken with a digital camera (DFC310FX; Leica Microsystems, Wetzlar, Germany) on a Leica DM5000B microscope (Leica Microsystems).

Double-label *in situ* hybridization for *esr1* mRNA and *vglut* or *gad* mRNA

We performed a double-label *in situ* hybridization using a mixture of DIG-labeled *esr1* and fluorescein-labeled *vesicular glutamate transporter* (*vglut*) *2.1*, *glutamic acid decarboxylase* (*gad*)*1.1*, *gad1.2* and *gad2* probes that were synthesized as described in a previous report (24). Medaka-cDNAs were PCR-amplified using a forward/reverse 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 fluorescein RNA labeling mixtures (Roche) according to the manufacturer's protocol.

After brains were fixed by transcardial perfusion, all procedures up to the blocking step were performed as described above. The sections were then 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 for 30 min with the Biotinyl Tyramide Amplification kit (TSA; NEL700A, PerkinElmer) diluted 1:50 in dilution buffer (PerkinElmer), washed with DIG-1 twice for 10 min each, and incubated with ABC reagents (Vector) for 1 h. Sections were washed twice with DIG-1 for 10 min then 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 using a 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. Sections were then coverslipped with CC/Mount (Diagnostic BioSystem). Fluorescent signals were observed and documented using a LSM-710 confocal laser-scanning microscope (Carl Zeiss). We calculated the percentage of co-expression between *esr1* expressing cells and *vglut* or *gad* expressing cells by counting the number of *esr1* expressing cells and both *esr1* and *vglut* or *gad* positive cells. Numbers of cells were expressed as mean ± SD.

Results

Histological analysis of the expression of Esr1 in GnRH1 neurons

We examined the expression of *esr1* in hypophysiotropic GnRH1 neurons in the preoptic area (POA) using *in situ* hybridization of adjacent sections (Fig.1 a-d). Abbreviations of brain nuclei referenced here are summarized in Table 3. We found that *esr1* mRNA was localized in the medial POA (Fig.1 c). On the other hand, *gnrh1* mRNA were localized in the lateral POA (Fig.1 d). Therefore, to determine if GnRH1 neurons express Esr1, we performed double *in situ* hybridization for *esr1* and *gnrh1*mRNA (Fig.1 e and f). We found 516±10 *esr1*-expressing cells and 32±4 *gnrh1*-expressing cells in the POA (means ± SD, n=2 fish). However, we did not find any co-expression of *gnrh1* and *esr1* mRNA (Fig.1 f). These results indicate that Esr1is not expressed in GnRH1 neurons.

Establishment of *esr1*:EGFP transgenic line

We generated a transgenic (Tg) medaka line that expresses EGFP specifically in Esr1 neurons (Fig.2 a-c). We found that the EGFP-positive cells were mainly distributed in the

supracommissural part of ventral telencephalic area (Vs), POA, and ventral tuberal nucleus (NVT) region, all of which have previously been reported to contain Esr-expressing neurons in medaka (20). To examine specificity, analyzed the results of double labeling using EGFP immunohistochemistry and *esr1* mRNA *in situ* hybridization (Fig.2 b and c). We found that 44% of POA-*esr1* neurons (201±23/460±51 cells, n=3 fish), and about 70% of Vs-*esr1* neurons (n=2) and NVT-*esr1* neurons (n=2) were labeled with anti-EGFP antibody. Although some EGFP-negative *esr1* cells were observed in these areas, all the EGFP positive cells expressed *esr1*, at least in these areas. Thus, the specificity of EGFP labeling is satisfactory for performing anatomical analysis of the axonal projections of these neurons.

In addition, we analyzed the results of double labeling analysis using EGFP immunohistochemistry and *esr2a*/*esr2b* mRNA *in situ* hybridization (Fig.2 d and e), because we have previously suggested that Esr1, Esr2a (or ERβ1) and Esr2b (or ERβ2) were widely distributed in POA, and that their distribution patterns in the POA were similar to each other. We found that EGFP also labeled *esr2a* and *esr2b* in this Tg line (Fig.2 d and e) and that 8% of POA-*esr2a* neurons (9±3/115±33 cells, n=2 fish) (Fig.2 d) and about 10% of POA-*esr2b* neurons (41±11/424±57 cells, n=2 fish) (Fig.2 e) were labeled with anti-EGFP antibody (see also Table4).

Anatomical analysis of the axonal projections of the Esr1 neurons

We performed EGFP immunohistochemistry in the brain of *esr1*:EGFP transgenic medaka to analyze axonal projections of Esr1 neurons (Fig.3). Immunohistochemistry for EGFP, which enhances EGFP signals, enabled precise morphological analysis of axonal projections. Most of the POA-Esr1 neurons projected their axons to the lateral POA. These fibers formed a thick bundle and projected caudally (Fig.3 b). Frontal sections show that fibers project caudally, passing through the ventrolateral region of the telencephalon and hypothalamus (Fig.3 c). In the hypothalamus, axons of the POA-Esr1 neurons coursed laterally to the anterior tuberal nucleus (NAT) and NVT. These bundles of fibers innervated the pituitary, and although these results do not exclude another possibility that some POA-Esr1 neurons project to other regions, we may safely conclude that the majority of axons of the POA-Esr1 neurons projected to the pituitary. Axons of Esr1 neurons entered the rostral pituitary and projected widely to the rostral half of the pituitary, whereas only a few EGFP immunoreactive fibers were observed in the caudal region (data not shown).

On the other hand, analysis of sagittal sections showed that Vs-Esr1 neurons mainly projected to caudal regions of the brain (Figs.4 a and b). Analysis of frontal sections showed that these EGFP positive axons of the Vs-Esr1 neurons projected caudally, passing near the midline region of the hypothalamus bilaterally (data not shown). These axons passed through the periventricular region of the hypothalamus, in the region lateral to periventricular posterior nucleus (Nppv), and coursed further caudally (data not shown). Finally, axons of

Vs-Esr1 neurons terminated in the region ventral to medial reticular formation (RFM) (Fig.4 c). In this Tg line, no EGFP-ir axon were observed projecting to the spinal cord.

Morphological analysis of axonal projections of Esr1 neurons to GnRH1 neurons

To assess the participation of Esr1 neurons in mediating estrogen feedback to GnRH1 neurons, we analyzed the results of double labeling using EGFP immunohistochemistry and *gnrh1* mRNA *in situ* hybridization (Fig.5). EGFP positive axons from POA-Esr1 neurons surrounded GnRH1-expressing neuronal cell bodies localized in the ventrolateral POA. Seventy-seven percent of GnRH1 neurons (30±6/39±6 cells, n=3 fish) appeared to receive axonal projections from POA-Esr1 neurons. On the other hand, very few axons were observed around the dorsal and medial groups of GnRH1 neurons, which have been suggested not to project to the pituitary (21,22,25).

Neurotransmitter candidates for the POA-Esr1 neurons

We next examined neurotransmitter candidates for POA-Esr1 neurons. First, we collected EGFP positive neurons from the POA of female *esr1*:EGFP transgenic medaka and performed RNA sequencing (RNA-seq) of those neurons. It should be noted that we found no trace of *kiss1* or *kiss2* gene expression, contrary to what we may expect from results in mammals (8,26). These results strongly support the results of previous *in situ* hybridization studies demonstrating that neither *kiss1* nor *kiss2* neurons are localized in the POA in medaka (11,12,27). Therefore, we examined the expression of marker genes for classical transmitters, glutamate and GABA. As markers for glutamatergic and GABAergic transmission, medaka has been reported to possess four subtypes of *vglut* (*vglut1.1*, *vglut1.2*, *vglut2.1*, *vglut2.2* and *vglut3*) and three subtypes of *gad* (*gad1.1*, *gad 1.2* and *gad2*), respectively (24). We evaluated the expression level of *vglut* and *gad* genes based on the average RPKM; using this approach, only *vglut2.1*, *gad1.1* and *gad2* were detected from the EGFP positive neurons (RPKM for *vglut2.1*: 2.13±3.9, *gad1.1*: 95.1±86, *gad2*: 23.6±14, others: RPKM<1). This result suggests that POA-Esr1 neurons include both glutamatergic and GABAergic neurons. Next, we analyzed co-localization of glutamatergic or GABAergic markers in the POA-Esr1 neurons by double *in situ* hybridization for mRNA of *esr1* and either *vglut2.1 gad1.1*, *gad1.2*, or *gad2* (fig.6). These results are summarized in Table5. More than 95% of POA-*esr1* neurons co-expressed *vglut 2.1* (Fig.6 b) (326±81/342±71 cells, n=3 fish). In addition, 36% of POA-*esr1* neurons co-expressed *gad 1.1* (Fig.6 c) (148±23/410±42 cells, n=2 fish). As most Esr1 neurons were shown here to be glutamatergic by double labeling of *esr1* and *vglut 2.1*, we did not examine other subtypes of *vglut*. Among the GABA synthesizing enzymes, *gad1.1* showed the highest percentage of co-localization with *esr1* mRNA (Fig.6). However, the co-localization ratio for *gad1.1* was much lower than that of *vglut2.1*, which suggests that glutamate is the main classical neurotransmitter in POA-Esr1 neurons. In addition, RNA-seq results suggested that EGFP-positive POA neurons from *esr1*:EGFP medaka express not only *esr1* (RPKM 188±81) but also *esr2a* and *esr2b* as well (RPKM 28.2±19 and 3.02±4.8). These

results are consistent with our histological results (Fig. 2 d and e). Moreover, among other sex steroid receptor genes, we found that POA-*esr1* neurons highly express *progesterone receptor* (*pr*) (RPKM 155±113). Therefore, we analyzed double labeling using EGFP immunohistochemistry and *pr* mRNA *in situ* hybridization. We found that about 68% of EGFP-expressing neurons in POA co-expressed PR (167±33/246±33 cells, n=3 fish) (Fig.7).

Discussion

In the present study, we generated transgenic medaka in which Esr1-expressing neurons are labeled with EGFP and examined the anatomy of these neurons. We identified the neuronal pathways mediating estrogen feedback signals to GnRH1 neurons as well as those mediating sex steroid-sensitive signals to a circuit that modulates sex behaviors. We found that POA-Esr1 neurons project their axons to GnRH1 neurons, and that the former comprise glutamatergic/GABAergic neurons, which may be widely conserved among mammals and teleosts. We also found a possible neural substrate for estrogen-sensitive modulation of sexual behaviors. Taken together with results of previous behavioral studies suggesting involvement of Vs neurons in the regulation of sexual behaviors (28-30), the present results suggest that the caudally projecting Esr1 neurons in Vs are involved in regulating sexual behaviors with respect to the level of circulating estrogen. In addition, we found that some POA-Esr1 neurons co-express PR, which suggests that progesterone, in addition to estrogen, may regulate reproduction and/or sexual behaviors by controlling POA-Esr1/PR neurons in accordance with serum hormone levels.

GnRH1 neurons do not express Esr1 in medaka.

In the present study, we analyzed the neuronal circuit(s) involved in transmission of estrogenic signals to GnRH1 neurons in medaka. Esr1 has been demonstrated to be an essential factor for regulation of the HPG axis in mammals (6,8). Therefore, we first examined whether or not GnRH1 neurons express Esr1 in medaka (Fig.1). Double *in situ* hybridization for *gnrh1* and *esr1* mRNA disproved the expression of Esr1 in GnRH1 neurons (Fig.1 f). Thus, as in mammals, GnRH1 neurons do not directly receive estrogenic signals via Esr1 in medaka (31,32).

Specificity of EGFP labeling in the brain of *esr1*:EGFP transgenic medaka

In the brains of *esr1*:EGFP transgenic medaka, we found that EGFP-labeled cell bodies were distributed in the POA, Vs, and NVT, and we demonstrated the specificity of EGFP labeling in these regions (Fig.2). It should be noted that not all of Esr1 neurons were labeled with EGFP (see Results); on the other hand, EGFP labeling was found in nucleus diffusus tori lateralis and the optic tectum, regions in which *esr1* mRNA was not detected by *in situ* hybridization. So far, it is technically impossible to determine whether this signal indicates ectopically labeled neurons or neurons labeled more sensitively than with *in situ* hybridization. Either way, these labeled neurons projected locally and did not interfere with

analysis of axonal projections of POA or Vs neurons in the present study. Interestingly, we also demonstrated, by double labeling of *esr2a/esr2b* mRNA *in situ* hybridization and EGFP immunohistochemistry, that some EGFP positive Esr1 neurons co-expressed Esr2a or Esr2b (Fig.2 d and e). This finding is consistent with RNA-seq results, which suggested that the Esr1 neuronal population includes Esr2a and/or Esr2b mRNA. Thus, a small population of Esr1 neurons in this Tg line can be considered to be Esr1/2a or Esr1/2b neurons.

Functions of POA-Esr1 neurons

Using our novel Tg medaka line, we obtained the first morphological evidence that glutamatergic/GABAergic POA-Esr1 neurons directly regulate hypophysiotropic GnRH1 neurons (Figs.5 and 6). Many close contacts were observed between the GnRH1 neuronal cell bodies and the nerve terminals of POA-Esr1 neurons, the vast majority of which were proven to be glutamatergic, and some of them were GABAergic. In medaka, a previous study demonstrated that expression of *esr1*, *esr2a* and *esr2b* in POA region was not changed by either ovariectomy or estrogen replacement (33). Thus, Esr1 is considered to be stably expressed in the POA and to regulate the release of glutamate or GABA in accordance with the serum estrogen level. Because glutamate and GABA are the major excitatory synaptic transmitters (GABA is also suggested to be excitatory to GnRH1 neurons; see 35-37), it follows that estrogen-dependent modulation of glutamatergic/GABAergic transmission may affect GnRH1 firing activity. In medaka, the firing activity of GnRH1 neurons changes towards puberty (34); therefore, it is possible that the system for regulating GnRH1 neuron activity changes during pubertal development. After sexual maturation, adult female medaka show daily spawning, and our previous study has documented time-of-day-dependent changes in the firing activity of GnRH1 neurons and in expression of LH and FSH (22). However, the neuronal mechanisms underlying puberty onset or time-of-day-dependent changes in GnRH1 neuronal activities have not yet been clarified. In mammals, glutamate/GABA is thought to play important roles in the regulation of puberty onset and the estrous cycle; the proportion of glutamatergic agonist AMPA-responsive GnRH1 neurons changes across puberty (35). Moreover, it has been reported that the number of fast synaptic transmissions (Glu/GABA) to GnRH1 neurons changes in accordance with the serum estrogen level in mammals (36,37). Finally, a recent study suggested that glutamatergic Esr1 neurons in the mouse limbic forebrain play a key role in the negative/positive steroid feedback regulation of GnRH neurons, while GABAergic Esr1 neurons play a role in the positive feedback (38). Thus, estrogen-dependent alteration of glutamatergic/GABAergic inputs may be important for the reproductive regulation of vertebrates in general, and the glutamatergic/GABAergic neurons that project to the GnRH1 neurons morphologically identified here in medaka are the candidates for such regulatory neurons.

In the present study, we also found that POA-Esr1 neurons co-express PR (fig.7). Interestingly, in mice, arcuate-GABA/PR neurons have been suggested to project heavily to

GnRH1 neurons (39). Results of the present study suggested that 36% of POA-Esr1 neurons are GABAergic. Thus, some POA-Esr1/PR neurons that express GABA likely project to GnRH1 neurons in medaka. Taken together with the results of previous study in mice, the innervation of GnRH1 neurons by GABA/PR neurons seems to be conserved among mammals and teleosts, and the GABA/PR neurons may be important for the regulation of the HPG axis in vertebrates. Medaka brains, even in adults, are small, and are advantageous for analyzing GFP-labeled POA-Esr1 neurons in whole brain *in vitro* preparations, in which we can maintain the neuronal circuitries regulating the GnRH1 neuron activities almost intact. Therefore, the Tg medaka we established in the present study should provide a good model system for studying estrogen-dependent changes in glutamatergic/GABAergic synaptic inputs that regulate GnRH1 neuron activities and hence the regulation of reproduction.

In addition to these neuronal circuits, mammals have developed a Kiss1-mediated HPG axis regulation system for the fine tuning of reproduction; e.g., follicular development and ovulation are suppressed during lactation in mammals, mainly due to the suppression of pulsatile GnRH/LH secretion (40). On the other hand, in teleosts, it has been recently demonstrated that GnRH is required for ovulation but not folliculogenesis (5), suggesting that glutamatergic innervation of GnRH1 neurons may be important for LH surges and ovulation rather than LH pulses. Interestingly, in a daily surge model of OVX+E mouse (41), spontaneous glutamatergic EPSPs from GnRH neurons drastically increases in the afternoon (36). These lines of evidence suggest that glutamatergic transmission may be important for LH surges in both species, although other possibilities cannot be excluded.

We also examined the projections from Esr1 neurons to the pituitary. Results of the present anatomical analysis suggested that POA-Esr1 neurons may directly regulate activity of gonadotropes. It should be noted, however, that axons of the Esr1 neurons were distributed broadly in the rostral half of the pituitary gland, which suggests that Esr1 neurons may also regulate the release of hormones other than gonadotropins. Further investigation using this transgenic line is necessary to clarify whether or not the POA-Esr1 neurons directly regulate gonadotropin release.

Regulation of Sexual behaviors by Vs-Esr1 neurons and POA- Esr1/PR neurons

Among rodents, estrogen is suggested to activate lordosis in female rats (18). However, the site of estrogen action in the neuronal circuits of this sexual behavior has not been clarified so far. In teleosts, previous studies reported that electrical stimulation of POA or Vs acutely induced sexual behavior in hime salmon (29). Thus, neurons in POA and Vs may be involved in the regulation of motor control of sexual behavior. In the present study, we showed that Vs-Esr1 neurons project as far as the ventral region of medulla. However, we did not find neural fibers of Vs-Esr1 neurons in the spinal cord in our Tg line. Therefore, uncharacterized neurons in the medulla that receive axonal projections from the Vs-Esr1 neurons may relay

this estrogenic modulation to the central pattern generator in the spinal cord that induces sexual behavior.

Our present results clearly demonstrated that some of the Esr1 expressing neurons in the POA co-express PR (Fig.7B), which is partly consistent with the results of previous studies in mice (42,43). In mice, it has been shown that ablation of Esr1/PR neurons in the ventromedial hypothalamus diminishes sexual behaviors (30). Progesterone is suggested to induce sexual behavior in teleosts as well as in mammals (44). As suggested in rodent studies, it is possible that these Esr1/PR neurons are involved in sexual behavior. It should be noted that an immunohistochemical study in mammals demonstrated that PRs were detected only in Esr1 neurons (43). However, we suggest that some PR neurons in medaka do not express Esr1. Although not all Esr1 neurons were labeled by EGFP in the Tg line we established here, as shown in Fig.7, many EGFP-negative PR neurons (326±44/460±42 cells, n=3 fish) were found in the POA. Thus, it is possible that PRs are expressed not only in Esr1 neurons but also in Esr1 negative neurons in medaka. The function of progesterone and PR is likely to differ between placental mammals and non-placental teleosts, and thus further studies using diverse mammals and teleosts are necessary to understand the reasons for this difference.

A recent study in mice suggested that optogenetic activation of Esr1 neurons in the ventrolateral subdivision of the ventromedial hypothalamus induced sexual behavior (45). Further investigation using genetic tools, such as Tg medaka that express optogenetic tools specifically in Esr1 neurons, may enable us to analyze neuronal circuits underlying estrogen-primed modulation of sexual behavior.

In conclusion, in the present study, we succeeded in anatomically identifying and visualizing two kinds of Esr1 neurons: glutamatergic and/or GABAergic Esr1 neurons in the POA that may relay gonadal estrogen feedback signals to GnRH1 neurons, and Esr1 neurons in the Vs that may modulate sexual behavior by acting on medullary circuits (Fig.8). The Tg medaka line established in the present study will contribute to our understanding of the estrogen regulation of reproduction and behavior in vertebrates.

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Fig. 1 GnRH1 neurons do not express Esr1 in medaka POA. (a), (b), Schematic illustrations of a lateral (left is rostral) (a) and frontal (b) sections of the medaka brain showing the plane of section corresponding to the photographs in (c), (d). The boxed area in the POA is shown in (c), (d). (c), (d), Light photomicrographs showing *in situ* hybridization for *esr1* mRNA (c) and *gnrh1* mRNA (d) in adjacent sections. These photographs show that GnRH1 neurons are localized in the region lateral to *esr1* neurons in POA. (e), Schematic illustrations of a frontal section of the medaka brain (e), showing the plane of section corresponding to the photographs in (f). The boxed area in (e) is shown in (f). (f), Photographs showing double labeling for *esr1* mRNA (*magenta*) and *gnrh1* mRNA (*green*), which demonstrate that POA GnRH1 neurons do not co-express Esr1. For abbreviations of brain nuclei, see Table 3. Scale bars: 25μm

Fig. 2 Establishment of *esr1*:EGFP transgenic medaka. (a), The construct used to generate the *esr1*: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, upstream of the first methionine of the *esr1* gene. This construct contains the *cardiac myosin light chain 2* (*cmlc2*) promoter region to express EGFP in the heart for screening. (b), Schematic illustrations of lateral (left is rostral) and frontal sections of the medaka brain, showing the plane of section corresponding to the panels in (c)-(e). The boxed area indicates the POA population of *esr1* neurons shown in (c); note that the midline is located in the center of the pictures in (c). The box in red indicates the POA population of *esr2a* and *esr2b* neurons shown in (d) and (e). (c), Photographs showing double labeling for EGFP immunohistochemistry (*green*) and *esr1* mRNA (*magenta*) in the POA, which demonstrates that EGFP specifically labeled Esr1 neurons. In addition to the POA, we confirmed specificity of EGFP labeling in the Vs and NVT, which also express Esr1 (data not shown). (d), Photographs showing double labeling for EGFP immunohistochemistry (*green*) and *esr2a* mRNA (*magenta*) in the POA, which demonstrates that EGFP labeled Esr2a neurons (arrowhead). Approximately 8% of Esr2a neurons were labeled by EGFP (9±3/115±33 cells, n=2 fish). (e), Photographs showing double labeling for EGFP immunohistochemistry (*green*) and *esr2b* mRNA (*magenta*) in the POA, which demonstrates that EGFP labeled Esr2b neurons (arrowhead). Approximately 10% of Esr2b neurons were labeled by EGFP (41±11/424±57 cells, n=2 fish). For the abbreviations of brain nuclei, see Table 3. Scale bars: 10μm.

Fig. 3 Light photomicrographs showing the sagittal (a, b) and frontal (c) sections of the *esr1*:EGFP neurons visualized with anti-EGFP antibody in the brain of female *esr1*:EGFP transgenic medaka. Sections were counter-stained with cresyl violet. (a), The boxed area in the schematic illustration (lateral view of the brain) is shown in (b). (b), Sagittal sections showing EGFP immunoreactive cell bodies in POA (arrowhead) and fiber bundles (arrow). Each photograph shows different mediolateral levels of the brain. Most fibers of the POA-Esr1 neurons project caudally and reach the ventral region of hypothalamus. (c), Frontal sections showing axons of the Esr1 neurons. The left column contains schematic drawings of the boxed areas in the photographs of the right column. POA-Esr1 neurons project their axons (arrows) through the lateral region of the POA and run caudally, passing the ventrolateral region of hypothalamus, and project to the pituitary (pit). For the abbreviations of brain nuclei, see Table 3. Scale bars: 100μm.

Fig. 4 Light photomicrographs showing sagittal (a, b) and frontal (c) sections of *esr1*:EGFP neurons visualized with EGFP antibody in the brain of female *esr1*:EGFP transgenic medaka. Sections were counter-stained with cresyl violet. (a), Sagittal section (right) showing EGFP immunoreactive (ir) cell bodies and axons. The boxed area in the schematic illustration (left, lateral view of the brain) is shown in the photographs in (b). Arrowheads indicate cell bodies in Vs region. The EGFP-ir neurons in Vs (Vs-EGFP-ir neurons) project their axons to the caudal region. (b), Sagittal sections more lateral to the photograph of (A), showing that axons from Vs region project caudally, which suggests that these fibers project to the medulla, passing through the diencephalon. Arrows indicate fibers that originate from Vs-Esr1 neurons. (c), Frontal section showing fibers of the Vs EGFP-ir neurons in the ventral region of medulla at the level indicated in the lateral view of the brain (top left). The boxed area in the schematic illustration (top right) is shown in the photograph below. Arrowheads indicate EGFP-ir axons. EGFP-ir fibers were not observed in more caudal regions. TE, telencephalon. For abbreviations of brain nuclei, see Table 3. Scale bars: 100μm (A, B), 50μm (c).

Fig. 5 Double labeling analysis using *esr1*:EGFP transgenic medaka suggests that POA-Esr1 neurons directly contact the cell bodies of the GnRH1 neurons. (a), Illustration of a frontal section of the medaka brain, showing the plane of section corresponding to the photographs in (b). The boxed area is shown in (b). (b), Photographs showing double labeling using EGFP immunohistochemistry (*green*) and *gnrh1* mRNA *in situ* hybridization (*magenta*) demonstrate that the cell bodies of GnRH1 neurons are surrounded by EGFP-ir fibers. Taken together with the distribution of axons of Esr1 neurons, POA-Esr1 neurons are suggested to make direct contacts on GnRH1 neurons. For abbreviations of brain nuclei, see Table 3. Scale bars: 25μm.

Fig. 6 Double *in situ* hybridization for *esr1* and marker genes for glutamate or GABA in POA. (a), Schematic illustration of a frontal section of the medaka brain, showing the plane of section corresponding to the photographs. The boxed area corresponds to the photographs in (b)-(e). (b), Photographs showing double labeling for *vglut2.1* mRNA (*green*) and *esr1* mRNA (*magenta*), demonstrating that POA-Esr1 expressing cells express *vglut2.1*. These results suggest that the majority of the POA-Esr1 neurons are glutamatergic. (c), Photographs showing double labeling for *gad1.1* mRNA (*green*) and *esr1* mRNA (*magenta*), which demonstrate that some POA-Esr1 expressing cells co-express *gad1.1*. About 36% of POA-Esr1 expressing neurons expressed *gad1.1* (148±23/410±42 cells, n=2 fish). These results suggest that most POA-Esr1 expressing neurons produce glutamate, and some POA-Esr1 expressing neurons produce GABA. (d), Photographs showing merged images of double *in situ* hybridization for *gad1.2* mRNA (*green*) and *esr1* mRNA (*magenta*), demonstrating that very few POA-Esr1 cells express *gad1.2*(arrowhead). (e), Photographs showing merged images of double *in situ* hybridization for *gad2* mRNA (*green*) and *esr1* mRNA (*magenta*), demonstrating that some POA-Esr1 cells express *gad2* (arrowhead). For abbreviations of brain nuclei, see Table 3. Scale bars: 25μm.

Fig. 7 Double labeling analysis using *esr1*:EGFP transgenic medaka suggests that POA-Esr1 neurons co-express progesterone receptor (PR). Photographs showing double labeling EGFP immunohistochemistry (*green*) and *pr* mRNA *in situ* hybridization (*magenta*), demonstrating that POA-Esr1 neurons express PR (arrowhead). Scale bars: 20μm.

Fig. 8 Illustration of a working hypothesis concerning Esr1-mediated regulation of reproduction and sexual behavior. Glutamatergic/GABAergic POA-Esr1 neurons receive estrogenic feedback signals and directly regulate GnRH1 neurons in accordance with the gonadal status. Glutamatergic/GABAergic synaptic transmission to GnRH1 neuron is altered by estrogen levels. Estrogen also acts on Vs-Esr1 neurons and possibly modulates expression of neurotransmitters or firing activity in accordance with the gonadal status. Unidentified neurons in the medulla that receive axonal projections from the Vs-Esr1 neurons are suggested to relay this estrogenic modulation to the motor pattern generator in the spinal cord to modulate sexual behavior. Although the projection has not been clarified, Esr1/PR neurons are also considered to be involved in the regulation of sexual maturation and sexual behaviors.

Table1. Antibody Table

Peptide/protein target	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used	RRID
EGFP	anti-EGFP antibody	Drs. Kaneko and Hioki (Kyoto University)	Rabbit; polyclonal	1:1000	AB_2716624

Table2.

gene	Ensembl gene ID
vglut1.1	ENSORLG00000011296
vglut1.2	ENSORLG00000007982
vglut2.1	ENSORLG00000006157
vglut2.2	ENSORLG00000005791
vglut3	ENSORLG00000011203
gad1.1	ENSORLG00000009208
gad1.2	ENSORLG00000017268
gad2	ENSORLG00000012966
esr1	ENSORLG00000014514
esr2a	ENSORLG00000017721
esr2b	ENSORLG00000018012
pr	ENSORLG00000002651

Table3.

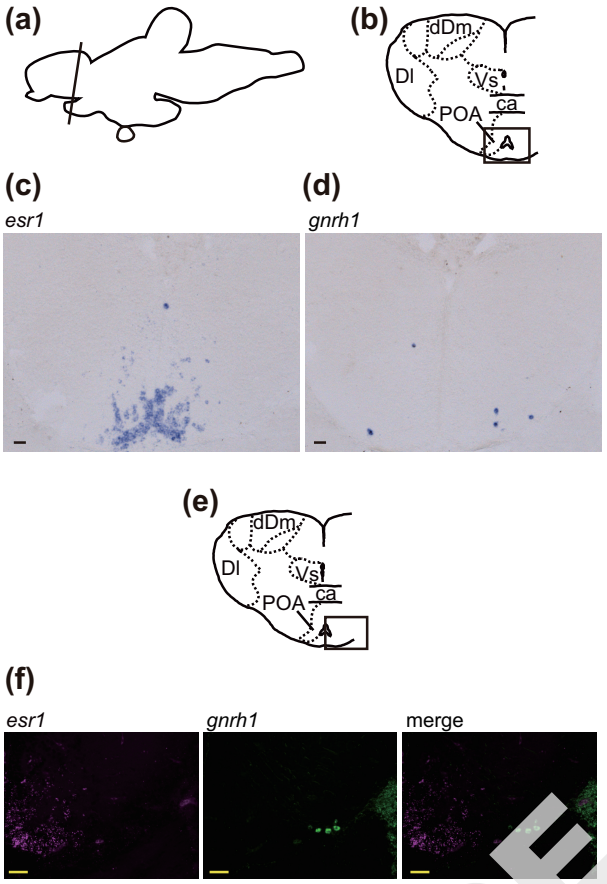
Abbreviations			
ca	anterior commissure	NVT	ventral tuberal nucleus
dDm	dorsal region of the medial part of dorsal telencephalic area	NPPv	periventricular posterior nucleus
dDl	dorsal region of Dl	POA	preoptic area
Dl	lateral part of dorsal telencephalic area	POm	magnocellular preoptic nucleus
		POp	parvocellular preoptic nucleus
DM	dorsomedial thalamic nucleus	RFm	medial reticular formation
Dp	posterior part of dorsal telencephalic area	TO	optic tectum
GR	corpus glomerulosus	TS	torus semicircularis
NAT	anterior tuberal nucleus	V	ventral telencephalic area
nII	optic nerve	VM	ventromedial thalamic nucleus
Dl	lateral part of dorsal telencephalic area	Vp	postcommissural part of V
		Vs	supracommissural part of V

Table4.

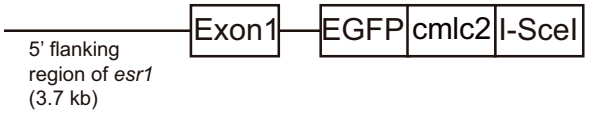
	Average number of EGFP labeled cells/*esr* neurons in POA	% of EGFP labeled cells
esr1	201±23/460±51 (n=3 fish)	44
esr2a	9±3/115±33 (n=2 fish)	8
esr2b	41±11/424±57 (n=2 fish)	10

Table5.

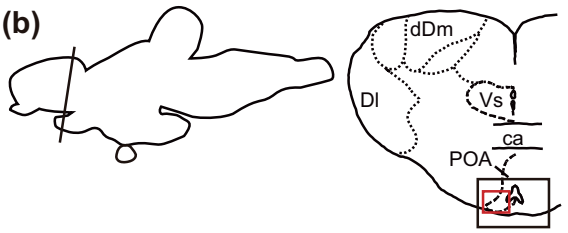
	Average number of cells in POA	% of each neurotransmitter- expressing POA-Esr1 neurons
vglut2.1 and *esr1*-expressing neuron	326±81 (in 342±71 Esr1 neurons, n=3 fish)	95
gad1.1 and esr1-expressing neuron	148±23 (in 410±42 Esr1 neurons, n=2 fish)	36
gad1.2 and esr1-expressing neuron	20±4 (in 515±28 esr1 neurons, n=2 fish)	4
gad2 and esr1-expressing neuron	110±18 (in 430±56 esr1 neurons, n=2 fish)	26



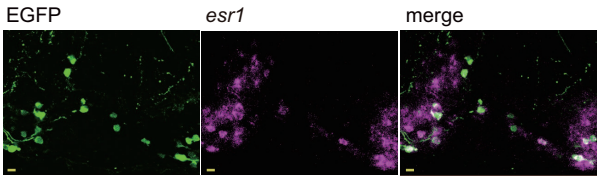
(a)



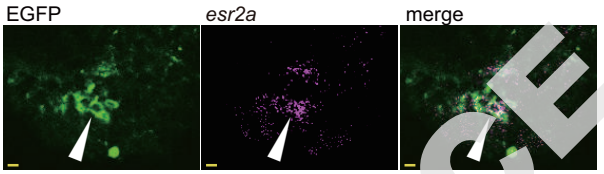
(b)



(c)



(d)



(e)