学位論文

Genes differentially expressed between the sexes in the medaka brain

（メダカの脳において発現に性差を示す遺伝子の探索と発現制御解析）

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Abstract

Vertebrates exhibit differences between sexes in a variety of behavioral and physiological traits. Many of these differences presumably reflect underlying sex differences in the brain. The majority of the studies on sexual differentiation of the vertebrate brain have employed rodent models. The results of these studies have repeatedly shown that androgen secreted by the testis early in life masculinizes the brain through conversion to estrogen locally in the brain and that this hormonal effect is irreversible and results in permanent sex differences in the brain. This process has been referred to as the general concept of sexual differentiation of the brain. However, several studies on non-rodent species, including humans, monkeys, and birds, have challenged this concept and provided evidence for alternative mechanisms for sexual differentiation of the brain. It has been shown, for example, that sex chromosome-linked genes, independent of gonadal hormones, also mediate sex differences in the brain.

The most obvious conflict with the above-described general concept has arisen from the studies in teleost fish showing that the teleost brain exhibits a considerable degree of sexual plasticity throughout lifetime. The phenotypic sex of teleosts, including sex-specific behavioral patterns, can be manipulated by hormonal treatment even after sexual maturity. More precisely, quite a few teleost species spontaneously undergo phenotypic sex reversal even in adulthood. These facts indicate that the above-mentioned concept that the brain is sexually differentiated in a permanent and irreversible manner during the early stage of life is by no means general in vertebrates. Teleosts undoubtedly have distinctive mechanisms of brain sexual differentiation to ensure life-long sexual plasticity. Studies in teleosts should thus provide insight into mechanisms underlying the plasticity and stability of sexual phenotype of the vertebrate brain. In addition, comparative approaches using teleosts should help uncover general principles of sexual differentiation of the vertebrate brain. However, very little has been known about sex differences in the teleost brain. Accordingly, in this thesis, which consists of three parts, I investigated the development, maintenance, and plasticity of sex differences in the brain of a teleost fish, medaka (*Oryzias latipes*).

In the first part (Chapter 1), I searched for genes differentially expressed between the

sexes in the medaka brain by microarray analysis. The result was that out of 63,698 profiled transcripts, 147 displayed male bias and 196 displayed female bias, with more than 3-fold differences in the relative levels of expression. I selected three transcripts with definite sex differences in expression (one female-biased and two male-biased transcripts) from them for further analysis. Verification of the differential expression of these three transcripts by real-time PCR revealed that they have approximately 16.1-fold female-biased and 5.6- and 3.6-fold male-biased expression. Determination of the full-length cDNA sequences followed by phylogenetic analyses revealed that the female-biased transcript encodes a member of the transglutaminase family, F13a1a, while the male-biased transcripts encode a polypeptide hormone belonging to the interleukin-6 family of cytokines, Cntfa, and a member of the PDZ-LIM protein family, Pdlim3a. Chromosomal mapping demonstrated that whereas *f13a1a* is located on an autosome, *cntfa* and *pdlim3a* are on sex chromosomes, in a region that is shared by the X and Y chromosomes.

In the second part (Chapter 2), I examined the spatiotemporal expression pattern of *f13a1a* and the mechanisms that caused its female-biased expression. I found that *f13a1a* is female-specifically expressed in two preoptic nuclei: the parvocellular portion of the magnocellular preoptic nucleus (PMp) and the magnocellular portion of the magnocellular preoptic nucleus (PMm), which have been implicated in controlling pituitary hormone secretion and sexual behavior, respectively. The expression of *f13a1a* was also seen in cells lying scattered immediately outside the brain parenchyma, which were much more numerous in females than in males. The female bias of *f13a1a* expression first appeared during puberty and increased markedly thereafter. Ovariectomy markedly decreased and estrogen replacement restores the *f13a1a* expression in the brain of adult females. In addition, estrogens induced its expression in the brain of adult males to a comparable level as in females. These results indicate that the sexually dimorphic expression of *f13a1a* results from stimulatory effects of estrogen secreted by the ovary and is completely reversible even in adulthood. Evidence was also obtained that the female-specific *f13a1a*-expressing neurons bear estrogen receptors and that an estrogen-responsive element-like sequence is present in the proximal promoter region of *f13a1a*, suggesting that *f13a1a* is a direct transcriptional target of estrogen and, as a consequence, preferentially expressed in females. The marked and

fully reversible sexual dimorphism in *f13a1a* expression, responding to gonadal estrogen, may possibly contribute to the development and reversal of sex differences in the brain and some physiological/behavioral traits.

The spatiotemporal expression pattern of *cntfa* and *pdlim3a* and the mechanisms underlying their male-biased expression were assessed in the last part of this thesis (Chapter 3). For both genes, comparable levels of expression with comparable levels of male bias were observed regardless of brain region, suggesting that they are expressed ubiquitously with unvarying sex differences in the brain. As opposed to *f13a1a*, sex differences in the expression of *cntfa* and *pdlim3a* were evident even before the onset of sexual maturity. Sex-reversed XY females, as well as wild-type XY males, had more pronounced expression of these genes than XX males and XX females, indicating that the Y allele confers higher expression than the X allele for both genes. In addition, their expression was affected to some extent by sex steroid hormones, thereby possibly serving as focal points of the crosstalk between the genetic and hormonal pathways underlying brain sex differences. Given that sex chromosomes of ectothermic vertebrates, including teleost fish, have evolved independently in different genera or species, sex chromosome genes with sexually dimorphic expression in the brain, like *cntfa* and *pdlim3a* in medaka as I showed here, may contribute to genus- or species-specific sex differences in a variety of traits.

Taken together, in this thesis I identified three novel genes (*f13a1a*, *cntfa*, and *pdlim3a*) that were differentially expressed between the sexes in the medaka brain, and elucidated part of the mechanisms behind the sex differences in their expression. Through the analysis of these genes, I found evidence, for instance, that 1) sexually dimorphic gene expression in the teleost brain is highly plastic even in adulthood, 2) sex differences exist not only in the brain parenchyma but also in its surrounding cells, 3) sex chromosome-linked genes exhibit sexually dimorphic expression in the brain, even in ectothermic vertebrate species, and 4) the expression of some sex chromosome genes is regulated both genetically and hormonally. These findings have provided insight into the mechanisms underlying sexual differentiation of the teleost brain, and also provided novel information on the plasticity and stability of sexual phenotype of the vertebrate brain. Additional studies, especially those examining the physiological roles of the genes identified in this thesis, will help further understand sexual

differentiation of the brain and physiological/behavioral traits in vertebrates.

General Introduction

Vertebrates exhibit differences between sexes in a wide range of behavioral and physiological traits, including reproductive behavior, aggression, food intake, sexual cycle, and stress response. Many of these differences presumably reflect underlying sex differences in the brain circuitry and other neural mechanisms. Indeed, a variety of anatomical and physiological sex differences have been identified in the vertebrate brain that could underlie differences in traits (although the specific overlaying behavior or physiology that these brain sex differences control is still unclear and largely speculative) (de Vries and Södersten, 2009; Forger, 2009; McCarthy and Arnold, 2011).

The vast majority of the studies on sexual differentiation of the vertebrate brain have been performed using rodents as models. The results of these studies have repeatedly shown that androgen secreted from the fetal testis reaches the brain of genetic males via blood, and is then converted to estrogen by the enzyme aromatase, which subsequently initiates the process of brain masculinization (Phoenix *et al*., 1959; Gorski and Wagner,1965; McDonald *et al*., 1970; MacLusky and Naftolin, 1981; Allen *et al*., 1989). On the other hand, when androgen concentrations are low, as occurs typically in genetic females, the development of the brain is feminine (rich in female-type characteristics). Importantly, the hormonal effect in this early developmental process is irreversible and causes permanent sex differences in the brain. These findings have been considered to represent the general pattern for sexual differentiation of the brain.

However, several studies in non-rodent species, including humans, monkeys, and birds, have provided evidence inconsistent with this generally accepted concept. For example, the application of androgen to pregnant rhesus monkeys altered the manner of sexual behavior in their female children to that of males, but the application of estrogen did not affect their sexual behavior (Pomerantz *et al*., 1986; Goy and Deputte, 1996). In humans, men with congenital aromatase deficiency displayed normal brain anatomy, gender identity, and sexual orientation (Morishima *et al*., 1995; Carani *et al*, 1997; 1999; Rochira *et al*, 2001). A man with estrogen resistance (the condition in which estrogen cannot be recognized and exert its biological action) also showed normal gender identity and sexual orientation (Smith *et al*.,

1994). These lines of evidence suggest that in primates including humans, aromatization of androgen to estrogen is not obligatory for masculinizing effects on the brain (androgen may directly affect masculinization (enrichment of male-type characteristics) of the brain).

Furthermore, evidence has recently been accumulating that sex chromosome complement, independent of gonadal hormones, also mediate sex differences in the brain (Arnold, 2009a; 2009b; Arnold and Chen, 2009; McCarthy and Arnold, 2011). This idea was established by a study on a naturally occurring lateral gynandromorphic finch, which was genetically male on the right side of its body and genetically female on the left side (Agate *et al*., 2003). Although both sides of the brain have been exposed to the same levels of gonadal hormones, the right side of the neural song circuit was more masculine than the left, indicating that sex differences in the brain is, at least in part, attributed to brain-autonomous factors encoded by sex chromosomes. This idea was further strengthened very recently by a study on artificially generated chicken chimeras in which brain primordia were exchanged between genetic males and genetic females prior to gonadal development (Maekawa *et al*., 2013). The female chimeras with male brains displayed delayed sexual maturation and irregular oviposition cycles, although their behavior, plasma concentrations of sex steroids and luteinizing hormone levels are normal.

The most obvious conflict with the concept developed by rodent studies has arisen from the studies on teleost fish showing that the teleost brain exhibits a considerable degree of sexual plasticity throughout lifetime. The phenotypic sex of teleosts, including sex-specific patterns of reproductive behavior, can be manipulated by treatment with exogenous hormones, even after sexual maturity (Howell *et al*., 1980; Stacey and Kyle, 1983; Stacey and Kobayashi, 1996; Paul-Prasanth *et al*., 2013; Takatsu *et al*., 2013). More precisely, a large number of teleost species spontaneously undergo phenotypic sex reversal in response to various social and physiological events, even in adulthood (Godwin, 2010). These facts indicate that teleosts have some mechanism of brain sexual differentiation that enables them to maintain marked sexual plasticity throughout their lifetime. Certainly, the well-accepted concept that the brain undergoes an irreversible process of sexual differentiation during the early stage of life cannot be applied to teleosts. However, very little is known about sexual differentiation and sex differences in the teleost brain.

Accordingly, in this thesis, I investigated the development, maintenance, and plasticity of sex differences in the teleost brain. I selected medaka (*Oryzias latipes*) as the model species for this study, because medaka offer several advantages as a model for sex-related studies. For example, medaka have an XX/XY sex-determining chromosomal system, while sex chromosomes have been identified in a small number of teleost species. Furthermore, medaka is one of a few teleost species in which the sex-determining gene has been identified (Matsuda *et al*., 2002; 2007; Nanda *et al*., 2002). The sex-determining gene of medaka, termed *dmy*, is located on the Y chromosome and is required for initiating the male sexual pathway of development, functionally comparable to the mammalian sex-determining gene *SRY*/*Sry* (Sinclair *et al*., 1990; Berta *et al*., 1990; Koopman *et al*., 1990). These characteristics facilitate the investigation of the genetic and hormonal aspects of sexual differentiation and the comparison of the results with those in mammals. In addition, phenotypic sex in medaka can be manipulated by hormonal treatment during both embryonic development and adulthood (Yamamoto, 1958; Paul-Prasanth *et al*., 2013).

This thesis consists of three parts. In the first part (Chapter 1), I searched for genes differentially expressed between the sexes in the medaka brain. This led to the identification of one female-biased gene, *f13a1a*, encoding a member of the transglutaminase family, and two male biased genes, *cntfa* and *pdlim3a*, encoding a polypeptide hormone belonging to the interleukin-6 family of cytokines and a member of the PDZ-LIM protein family, respectively. In the second part (Chapter 2), I investigated the spatiotemporal expression pattern of *f13a1a* and the mechanisms underlying the sex difference in its expression. In the third part (Chapter 3), I performed the same analyses for *cntfa* and *pdlim3a*. The data obtained in this thesis uncovered several novel mechanisms underlying sex differences in gene expression in the teleost brain, and also provided insights into sexual differentiation of the vertebrate brain.

Chapter 1

Searching for genes differentially expressed between the sexes in the medaka brain

Introduction

As described in General Introduction, studies in teleost fish should help understand general phenomena across vertebrates and further define species- or class-specific aspects relating to sexual differentiation of the brain. However, very little is known about the mechanisms underlying this process in teleosts–in actual fact, limited information is available even about what sex differences exist in the brain in teleosts. Sex differences in the teleost brain have been reported only for the expression of several genes encoding neurohormone- and neurotransmitter/neuromodulator-related molecules. These genes includes gonadotropin-releasing hormone (Gnrh) (Elofsson *et al*., 1997, 1999; Ishizaki *et al*., 2004; Maruska *et al*., 2007; Kuramochi *et al*., 2011; Kawabata *et al*., 2012), kisspeptin (Kiss) (Kanda *et al*., 2008), vasotocin (Vt) (Grober and Sunobe, 1996; Foran and Bass, 1998; Godwin *et al*., 2000; Grober *et al*., 2002; Ohya and Hayashi, 2006; Maruska *et al*., 2007; Maruska, 2009; Kawabata *et al*., 2012), isotocin (It) (Black *et al*., 2004; Kawabata *et al*., 2012), galanin (Cornbrooks and Parsons, 1991a; 1991b; Rao *et al*., 1996; Jadhao and Meyer, 2000; Rodríguez *et al*., 2003), aromatase (Borg *et al*., 1987; Pasmanik and Callard, 1988; Melo and Ramsdell, 2001; González and Piferrer, 2003; Goto-Kazeto *et al*., 2004; Strobl-Mazzulla *et al*., 2005; Patil and Gunasekera, 2008), tyrosine hydroxylase (Th) (Chaube and Joy, 2003), and tryptophan hydroxylase (Tph) (Sudhakumari *et al*., 2010; Raghuveer *et al*., 2011; Kawabata *et al*., 2012).

Quite recently, our research group screened for genes exhibiting sexually dimorphic patterns of expression in the brain of medaka by performing suppression subtractive hybridization. This screening resulted in the identification of a dozen genes with male- or female-biased expression in the medaka brain, including *cyp19a1b* (encoding aromatase) (Okubo *et al*., 2011), *hebp3* (encoding a novel member of the heme-binding protein family) (Nakasone *et al*., 2013), and *npb* (encoding neuropeptide B) (Hiraki *et al*., 2014). However,

this approach may have left many possible genes unnoticed, and a larger-scale screening would expectedly lead to the identification of more genes that are differentially expressed between the sexes in the medaka brain.

Hence, in this chapter, I utilized a microarray-based approach to identify such genes. This approach indeed did lead to the identification of three novel sexually dimorphic genes, *f13a1a*, *cntfa*, and *pdlim3a*.

Materials and Methods

Animals

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Tokyo. Medaka of the Qurt strain were used in the microarray screening, and those of the d-rR strain were used in real-time PCR and cDNA cloning. They were maintained at 28°C with a 14-hour light/10-hour dark photoperiod and were fed 3 or 4 times per day with live brine shrimp and commercial pellet food (Otohime; Marubeni Nisshin Feed, Tokyo, Japan). Sexually mature adults of 3–7 months of age were sampled at 0.5–3 hours following the onset of light and used for analyses.

Microarray-based screening

A total of 617,608 medaka transcript sequences were obtained from publicly available databases, including the nucleotide database of the National Center of Biotechnology Information (NCBI) (286,247 sequences), the medaka transcript dataset from Ensembl (24,662 sequences), The Institute for Genomic Research (TIGR) medaka gene index (19,336 sequences), and the medaka expressed sequence tag (EST) database at the National BioResource Project (NBRP) (265,859 sequences), and the EST dataset derived from the medaka adult brain cDNA library constructed by our research group (Okubo *et al*., 2011) (21,504 sequences). These sequences were assembled using the CAP3 program (Huang and Madan, 1999). Redundant sequences were removed with a threshold of 95% identity in a 200-bp window using BLAST (Altschul *et al*., 1990) and custom Perl scripts. This resulted in

a dataset comprising 63,698 non-redundant sequences. A high-density oligonucleotide microarray containing 385,000 probes (60-mer probes) representing this set of non-redundant medaka transcripts was designed and manufactured by Roche Diagnostics Japan (Tokyo, Japan).

Total RNA was isolated from the whole brain along with the pituitary of male and female medaka (n = 25 for each sex) using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using the SuperScript Double-Stranded cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA) and labeled by random priming using Cy3-labeled random nonamers.

Microarray hybridization, washing, scanning, and data acquisition were performed by Roche Diagnostics Japan according to their standard protocols (NimbleGen Arrays User's Guide: Gene Expression Analysis v3.2). Data were normalized using a Robust Multichip Average (RMA) algorithm using the NimbleScan software (Roche Diagnostics, Basel, Switzerland).

Verification of differential expression by real-time PCR

Differential expression of several transcripts that were identified by microarray profiling was examined using real-time PCR. Total RNA was isolated from the whole brain of male and female medaka (n = 4 for each sex) using the RNeasy Plus Universal Mini Kit (Qiagen). Complementary DNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). PCR was performed using LightCycler 480 SYBR Green I Master on a LightCycler 480 System II (Roche). A melting curve analysis was conducted for every reaction to ensure that a single amplicon was produced in each sample. The expression of β-actin (*actb*) in each sample was used to normalize the expression of target transcripts by the standard curve method. The primers used for real-time PCR are listed in Table 1-1.

cDNA cloning

I selected three transcript sequences (ID: M26225, M50209, and M25981) that were confirmed by real-time PCR analysis to have definite sex differences in expression for further analysis. First the full-length sequences of these transcripts were determined as described

below. Primers used for this procedure are listed in Table 1-1.

The sequence M26225 was derived from an EST clone of NBRP (NBRP clone ID: olovano41_m05; GenBank accession numbers: DK196357/DK219412), which was fully sequenced by NBRP. The sequence of this clone was subjected to BLAST searches against public databases and found to represent the 3′ fragment of an NCBI-predicted transcript (GenBank accession number: XM_004080861). The remaining 5′ sequence was isolated by reverse transcription-PCR (RT-PCR) and 5′-rapid amplification of cDNA ends (RACE) from whole brain poly $(A)^+$ RNA using the Marathon cDNA Amplification Kit (Takara Bio, Shiga, Japan). The resulting PCR products were ligated into the pGEM-Teasy vector (Promega, Madison, WI, USA) and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) on Applied Biosystems 3730xl DNA/3130xl Genetic Analyzers (Life Technologies).

A partially sequenced EST clone of NBRP (GenBank accession numbers: BJ014070/BJ028232), from which the sequence M50209 was derived, was obtained from NBRP and fully sequenced as described above. On performing sequencing and BLAST analyses, this clone was also found to represent the 3′ fragment of an NCBI-predicted transcript (GenBank accession number: XM_004065858). The remaining 5′ sequence was obtained by 5′-RACE with the Marathon cDNA Amplification Kit (Takara Bio) as described above.

BLAST searches of public databases using an EST clone of NBRP (GenBank accession numbers: BJ712867/BJ724311), from which the sequence M25981 was derived, as the query identified several longer, full-length EST clones containing this sequence in the NBRP database (http://www.shigen.nig.ac.jp/medaka/). The longest clone (NBRP clone ID: olsp16d13), which was fully sequenced by NBRP, was selected as the representative, full-length transcript corresponding to the sequence M25981.

Phylogenetic analysis

The resultant full-length medaka transcripts containing the sequences M26225, M50209, and M25981 had top BLAST hits to *F13a1*/*f13a1*, *Cntf*/*cntf*, and *Pdlim3*/*pdlim3*, respectively, in other vertebrate species. The deduced amino acid sequences of these transcripts were

aligned with known and predicted sequences of F13A1/F13a1, CNTF/Cntf, and PDLIM3/Pdlim3 in other species using ClustalW (Thompson *et al*., 1994). The resulting alignment was used to construct a bootstrapped (1,000 replicates) neighbor-joining tree (http://clustalw.ddbj.nig.ac.jp/index.php). Transglutaminase 1 (TGM1) and TGM2 in humans and mice were used as outgroups for the F13A1/F13a1 tree. Cardiotrophin-like cytokine factor 1 (CLCF1), cardiotrophin 1 (CTF1), and leukemia inhibitory factor (LIF) in humans and mice were used as outgroups for the CNTF/Cntf tree. PDLIM1, PDLIM2, and PDLIM4 in humans and mice were used as outgroups for the PDLIM3/Pdlim3 tree. The species names and accession numbers of the sequences that were used in the analysis are listed in Tables 1-2, 1-3, and 1-4.

Chromosomal mapping

The chromosomal positions of *f13a1a* (containing the sequence M26225), *cntfa* (containing the sequence M50209), and *pdlim3a* (containing the sequence M25981) were mapped using the Ensembl BLAST server (http://www.ensembl.org/Multi/blastview). Several genes that were reported to be located adjacent to the Y-specific region, which contained the sex-determining gene *dmy*, on the medaka sex chromosome, including *abcg2*, *pkd2*, *vdp*, *herc3*, and *fam13a1* (Kondo *et al*., 2006), were also mapped in the same manner.

Statistical analysis

Data obtained from real-time PCR are presented as the mean and the standard error of the mean. The expression level of target transcripts (normalized to that of *actb*) in the male brain was arbitrarily set to 1, and that in the female brain was calculated relative to it. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Comparisons between the data from males and females were evaluated for statistical significance by the unpaired two-tailed Student's *t*-test.

Results

Identification of sexually dimorphic transcripts in the medaka brain

Microarray analysis was performed to identify genes that were differentially expressed between the sexes in the medaka brain. Of 63,698 profiled transcripts, 147 (0.23%) and 196 (0.31%) displayed male bias and female bias, respectively, with more than 3-fold differences in the relative levels of expression (Figure 1-1). These differentially expressed transcripts included *npb*, which has been identified as being expressed in a female-biased manner (about 10-fold higher in females) in the medaka brain by a subtractive screen (Hiraki *et al*., 2014).

In this thesis, I focused on one female-biased transcript (ID: M26225), which displayed 28.1-fold higher expression in females than in males, and two male-biased transcripts (ID: M50209 and M25981), which displayed 3.7- and 3.1-fold higher expression, respectively, in males than in females. The differential expression of these transcripts between the sexes was verified by real-time PCR, in which M26225 exhibited 16.1-fold higher expression in females, while M50209 and M25981 showed 5.6- and 3.6-fold higher expression, respectively, in males (Figure 1-2).

Structural and phylogenetic characterization of the transcripts identified in the screen

The full-length sequences of the transcripts corresponding to M26225, M50209, and M25981 were determined by using *in silico* analysis, RT-PCR, and RACE. The nucleotide and deduced amino acid sequences of these transcripts are shown in Figures 1-3, 1-4, and 1-5. BLAST search revealed that the proteins encoded by M26225, M50209, and M25981 were most similar to known F13A1/F13a1, CNTF/Cntf, and PDLIM3/Pdlim3, respectively (Figures 1-6, 1-7, and 1-8). A gene encoding F13a1 has already been identified in medaka (Koh *et al*., 2004), but the sequence of M26225 was found to differ considerably from that of this gene.

Phylogenetic analysis demonstrated that M26225, M50209, and M25981 did indeed encode a medaka homolog of F13A1/F13a1, CNTF/Cntf, and PDLIM3/Pdlim3, respectively, in other vertebrate species (Figures 1-9, 1-10, and 1-11). Three paralogous genes encoding F13a1, including a predicted gene in the genome, have been identified in zebrafish (Deasey *et al*., 2012). M26225 was found to encode a medaka ortholog of the two F13a1 proteins in zebrafish (GenBank accession numbers: NP_001070179 and XP_686649) and they were

paralogous to the other F13a1 in zebrafish (NP_001070622) and the previously identified medaka F13a1 (NP_001098300). Accordingly, the former orthologous group in the teleost lineage (including the products of the medaka transcripts that were identified in this thesis) was designated F13a1a and the latter, F13a1b (Figure 1-9). Two paralogs were also present for both Cntf and Pdlim3 in the teleost lineage; accordingly, they were designated Cntfa/Cntfb and Pdlim3a/Pdlim3b (the products of the medaka transcripts that were identified in this thesis were designated Cntfa and Pdlim3a) (Figures 1-10 and 1-11).

The sequence of the medaka *cntfa* was deposited in GenBank under accession number AB894420.

Chromosomal location of *f13a1a*, *cntfa*, and *pdlim3a*

f13a1a was mapped to chromosome 20 (an autosome) at location 1,803,711–1,812,336. On the other hand, both *cntfa* and *pdlim3a* were mapped to the chromosome 1 (sex chromosome). A comparison of their locations relative to those of several sex chromosome-linked genes residing adjacent to the Y-specific region, which contained the sex-determining gene *dmy*, revealed that *cntfa* and *pdlim3a* were located 3–4 Mb from and on opposite sides of the Y-specific region (Figure 1-12).

Discussion

The study described in this chapter was aimed at exploring sex differences in gene expression in the medaka brain. In an attempt to achieve the same aim, a differential screen using suppression subtractive hybridization has recently been conducted by our research group, resulting in the identification of a dozen of sexually dimorphic genes from approximately 6,000 redundant clones examined (Okubo *et al*., 2011; Nakasone *et al*., 2013; Hiraki *et al*., 2014). By employing a larger-scale, microarray-based screening approach, here I extended the list of sexually dimorphic genes in the medaka brain to include *f13a1a*, *cntfa*, and *pdlim3a*.

Phylogenetic analyses revealed that these genes are one of two teleost lineage-specific paralogs encoding F13a1, Cntf, and Pdlim3, which presumably arose from a whole-genome

duplication early in teleost evolution (Amores *et al*., 1998). Following the widely accepted nomenclature for duplicated genes in teleosts (ZFIN Zebrafish Nomenclature Guidelines), these genes were designated with the suffix "a". While the other medaka paralog for F13a1 has been identified (previously called embryonic transglutaminase (*em*TGase) but here renamed *f13a1b*) (Koh *et al*., 2004), those for Cntf and Pdlim3 have not been identified to date and, moreover, could not be found in the medaka genome database; therefore, *cntfb* and *pdlim3b* were likely lost in medaka during evolution.

F13A1, also called coagulation factor XIII (FXIII) A subunit, is a member of the transglutaminase family and, when activated, catalyzes the formation of covalent cross-links between glutamine and lysine residues in proteins. FXIII is found extracellularly in plasma and intracellularly in a variety of cell types, including platelets, monocytes/macrophages, granulocytes, hepatocytes, chondrocytes, and osteoblasts/osteocytes (Muszbek *et al*., 2011; Richardson *et al*., 2013). In plasma, FXIII circulates in the form of a tetramer composed of two catalytic A subunits (F13A1) and two carrier B subunits (F13B), whereas intracellular FXIII consists of two A subunits (F13A1) only. F13A1 is best known for its role in blood coagulation. In the last step of the coagulation cascade, F13A1 stabilizes the fibrin clot by covalently cross-linking fibrin monomers and antifibrinolytic proteins. In addition to these blood coagulation-related substances, F13A1 is able to cross-link other proteins and has been implicated in a variety of physiological and pathological processes, including, for example, maintenance of pregnancy, wound healing, vascular remodeling, angiogenesis, tissue repair, cartilage and bone development, recurrent spontaneous abortion, thrombosis, and atherosclerosis (Muszbek *et al*., 2011; Richardson *et al*., 2013; Levy and Greenberg, 2013).

Despite these findings in mammals, there have been only a few studies addressing F13a1 in teleosts. The expression of *f13a1b* was observed in the anterior lateral plate mesoderm and yolk veins in medaka embryos (Koh *et al*., 2004). In zebrafish, three F13a1-coding genes, which were found here to be two *f13a1a* and one *f13a1b*, have been identified, and the expression of *f13a1b* was detected in the developing pectoral fin (Deasey *et al*., 2012). To date, only one study has investigated F13a1 function in teleosts (Sugitani *et al*., 2012). This study has shown that, after optic nerve injury, F13a1 expression was upregulated in astrocytes/microglial cells in the optic nerve and retinal ganglion cells and that overexpression

of F13a1 in retinal ganglion cells promoted neurite outgrowth in these cells, suggesting a role for teleost F13a1 in tissue repair as has been reported for mammalian F13A1 (Sugitani *et al.*, 2012).

To the best of my knowledge, *f13a1a* exhibits larger sex difference (16.1-fold) in expression at the whole brain level than any other genes in any vertebrate species, except sex chromosome-linked genes that are present and expressed only in one sex. This finding implies that *f13a1a* may play a major role in the context of sex differences in the brain. This is precisely why I chose *f13a1a* as a target for my study. The detailed expression pattern and regulatory mechanisms for this gene was investigated and described in the next chapter.

The other two genes, *cntfa* and *pdlim3a*, were chosen as targets for my study, mainly because these genes were found to be located on sex chromosomes. While previous studies have identified several sex chromosome-linked genes that were differentially expressed between the male and female brain in mammals and birds, there have been no reports of any sex chromosome-linked genes with sexually dimorphic expression in the brain of reptiles, amphibians, and fish (for details, see Chapter 3). It seemed plausible to expect that further analysis of *cntfa* and *pdlim3a* might shed light on some general and specific aspects of sexual differentiation of the vertebrate brain, especially the direct effects of sex chromosomes on this process. This expectation led me to examine the detailed expression pattern and regulatory mechanisms for *cntfa* and *pdlim3a* (which is described in Chapter 3).

CNTF/Cntf is a polypeptide hormone belonging to the interleukin-6 family of cytokines. CNTF, which has been originally described as a survival factor for neurons (Adler *et al.*, 1979), has been shown to play essential roles in a variety of processes in the nervous system, including the self-renewal and differentiation of neural stem cells during embryonic development and in the adult brain in normal and diseased/injured states (Sleeman *et al.*, 2000; Bauer *et al.*, 2007). At present, little is known about Cntf in teleosts except that a *cntf*-like gene has been cloned in rainbow trout (Wang and Secombes, 2009), which was found to be *cntfb*, one of the two *cntf* paralogs occurring in teleosts, by the phylogenetic analysis used in the present study.

PDLIM3/Pdlim3, also known as actinin-associated LIM protein (ALP), is a member of the PDZ-LIM protein family defined by an N-terminal PDZ domain and one or three

C-terminal LIM domains (te Velthuis and Bagowski, 2007; Krcmery *et al*., 2010). This protein was shown to be essential for the development of cardiac muscle (Pashmforoush *et al*., 2001; Lorenzen-Schmidt *et al*., 2005). Although the expression of zebrafish *alp* and *alp-like*, which were found to be *pdlim3a* and *pdlim3b*, respectively, in the present study, was detected in the brain during embryonic development (te Velthuis *et al*., 2007), no information is available about its role in the brain in any species.

In summary, in this chapter, I searched for genes differentially expressed between the sexes in the medaka brain by a microarray-based approach and identified three genes, *f13a1a*, *cntfa*, and *pdlim3a*, whose sexually dimorphic expression has never previously been reported in any species. It may be of interest to see whether the sexually dimorphic expression of these genes is conserved among vertebrates or specific to teleosts (or even to medaka only). *f13a1a* exhibited the greatest degree of sexually dimorphic expression ever found in the brain. *cntfa* and *pdlim3a* represented the first examples of sex chromosome genes with sexually dimorphic expression in the brain in ectothermic vertebrates. Owing to these features, *f13a1a*, *cntfa*, and *pdlim3a* seemed to be worth further investigation and this was undertaken in the next chapters.

Table 1-1. Primers used in Chapter 1.

Target	Direction	Purpose	Sequence (5′ to 3′)
f13a1a	Forward	RT-PCR	GCCAAGAGTTCGCCATAAACATCA
f13a1a	Reverse	RT-PCR	GGCATGTACTCCTGAGGCAAGA
f13a1a	Reverse	Primary PCR of 5′-RACE	GCGATGGCCACATAGGTGCGATACA
f13a1a	Reverse	Nested PCR of 5′-RACE	GTGTCCGGTGTGATTCCAACTGTCGT
f13a1a	Forward	Real-time PCR	CCTGGACCACAGGGAGTTTAAG
f13a1a	Forward	Real-time PCR	AGTACAGCCACGATGCTCCTC
cntfa	Forward	Real-time PCR	AGAAGTGCTCGACGATGAGAAG
cntfa	Reverse	Real-time PCR	GGCATGGAGCGCAGTAATTT
cntfa	Reverse	Primary PCR of 5′-RACE	CCTCGCTGAAGACCTGAAGCAGCCA
cntfa	Reverse	Nested PCR of 5′-RACE	CAGCCATAGCTTCAGCTGAAACAGAGT
pdlim3a	Forward	Real-time PCR	AACATCCAAGATGCCATGGAG
pdlim3a	Reverse	Real-time PCR	CACGTCAGATTCCTCGATGCT
actb	Forward	Real-time PCR	GCCTCAAAGCTATTATTTTACTCAACT
actb	Reverse	Real-time PCR	GATGTAATGAATGAGTGCGTCTG

RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA ends.

Table 1-2. Species names and accession numbers of the protein sequences used for phylogenetic analysis of F13A1/F13a1.

Protein	Species	Accession number
F13A1	Human (*Homo sapiens*)	NP_000120
F13A1	Bovine (*Bos taurus*)	NP_001161366
F13A1	Mouse (*Mus musculus*)	NP_001159863
F13A1	Rat (*Rattus norvegicus*)	NP_067730
F13a1	Chicken (*Gallus gallus*)	NP_990016
F13a1a (predicted)	Tilapia (*Oreochromis niloticus*)	XP_003452423
F13a1a (predicted)	Fugu (*Takifugu rubripes*)	XP_003978009
F13a1a1	Zebrafish (*Danio rerio*)	NP_001070179
F13a1a2 (predicted)	Zebrafish (*Danio rerio*)	XP_686649
F13a1b (predicted)	Fugu (*Takifugu rubripes*)	XP_003975980
F13a1b	Medaka (*Oryzias latipes*)	NP_001098300
F13a1b	Zebrafish (*Danio rerio*)	NP_001070622
F13a1b	Salmon (*Salmo salar*)	NP_001167116
TGM1	Human (*Homo sapiens*)	NP_000350
TGM1	Mouse (*Mus musculus*)	NP_001155186
TGM2	Human (*Homo sapiens*)	NP_004604
TGM2	Mouse (*Mus musculus*)	NP_033399

Table 1-3. Species names and accession numbers of the protein sequences used for phylogenetic analysis of CNTF/Cntf.

Protein	Species	Accession number
CNTF	Human (*Homo sapiens*)	NP_000605
CNTF	Mouse (*Mus musculus*)	NP_740756
Cntf	Chicken (*Gallus gallus*)	NP_990823
Cntfa (predicted)	Zebra mbuna (*Maylandia zebra*)	XP_004564405
Cntfb (predicted)	Zebra mbuna (*Maylandia zebra*)	XP_004554543
Cntfb (predicted)	Tilapia (*Oreochromis niloticus*)	XP_003450378
Cntfb	Rainbow trout (*Oncorhynchus mykiss*)	NP_001165325
Cntfb	Zebrafish (*Danio rerio*)	NP_001139104
CLCF1	Human (*Homo sapiens*)	NP_037378
CLCF1	Mouse (*Mus musculus*)	NP_064336
CTF1	Human (*Homo sapiens*)	NP_001321
CTF1	Mouse (*Mus musculus*)	NP_031821
LIF	Human (*Homo sapiens*)	NP_002300
LIF	Mouse (*Mus musculus*)	NP_032527

Table 1-4. Species names and accession numbers of the protein sequences used for phylogenetic analysis of PDLIM3/Pdlim3.

Protein	Species	Accession number
PDLIM3	Human (*Homo sapiens*)	NP_001107579
PDLIM3	Monkey (*Macaca mulatta*)	NP_001253629
PDLIM3	Bovine (*Bos taurus*)	NP_001029818
PDLIM3	Mouse (*Mus musculus*)	NP_058078
Pdlim3a	Zebrafish (*Danio rerio*)	NP_001019547
Pdlim3b	Zebrafish (*Danio rerio*)	NP_001036183
PDLIM1	Human (*Homo sapiens*)	NP_066272
PDLIM1	Mouse (*Mus musculus*)	NP_058557
PDLIM2	Human (*Homo sapiens*)	NP_789847
PDLIM2	Mouse (*Mus musculus*)	NP_666090
PDLIM4	Human (*Homo sapiens*)	NP_003678
PDLIM4	Mouse (*Mus musculus*)	NP_062290

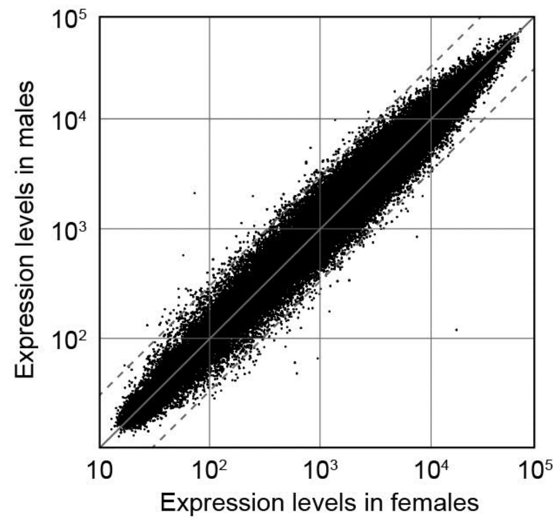

Figure 1-1. Microarray-based screening of transcripts with sex-biased expression in the medaka brain. Scatter plot comparing the expression profiles that were obtained by microarray analysis with the male (Y-axis, log scale) and female (X-axis, log scale) brain. Each dot represents one transcript (a set of probes) on the array. The solid diagonal line indicates no difference between the male and female brain, and the dashed lines indicate the three-fold differences. The perpendicular distance of a point from the solid diagonal line represents the degree to which a transcript is differentially expressed between the sexes.

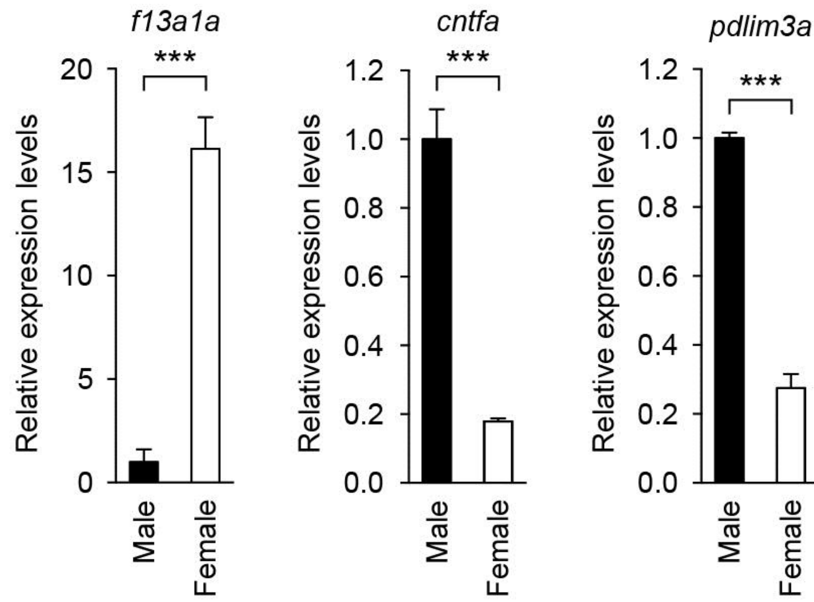

Figure 1-2. Verification of the sexually dimorphic expression of the transcripts identified in the screen. The expression of three transcripts, M26225 (*f13a1a*), M50209 (*cntfa*), and M25981 (*pdlim3a*), in the medaka brain was assessed by real-time PCR. ***, $p < 0.001$ (unpaired *t*-test).

```
GAAGCCCAGCAGACCTCTCCTCTCCTCTTCATCACTGCGCTCCTGTTCGTGCAGCCAAACCTTAAGCTTCTGTCACCATGAGGAAGGCCCAGCTGAGCAT
                                                                              M  R  K  A  Q  L  S  M
GAAAGGCCGCTTCCAACGGCCGGTGCCGACCTCCAACCTTCACCTGGAGGATGAGGCAGATTACAGAGAGTTTGAGCCATTTGAAAGGGATGTGGGTTTC
 K  G  R  F  Q  R  P  V  P  T  S  N  L  H  L  E  D  E  A  D  Y  R  E  F  E  P  F  E  R  D  V  G  F
TTCCCCAGAGCGCCTGCAGGCGACGATGGAAGCGCTCTGCAGGTGATGACCATTGATATGCAGAAGGACCAGAATGCTGGGAGCCACAACACCGTAGACT
 F  P  R  A  P  A  G  D  D  G  S  A  L  Q  V  M  T  I  D  M  Q  K  D  Q  N  A  G  S  H  N  T  V  D
ACATCAACACACCACTGCTGGTGGTCCGCCGCGGCCAAGAGTTCGCCATAAACATCACCTTCAACCGCCCACTGGCACCAGAAGATGATGTGCAGATCGA
Y  I  N  T  P  L  L  V  V  R  R  G  Q  E  F  A  I  N  I  T  F  N  R  P  L  A  P  E  D  D  V  Q  I  E
GTTCCTGATCGGTTCTGACCCAACCCCCCAGAAGAGATCCCTGCAGATCGTGACTTTGGGTAACCGGGAGGGCGGCACCTGGAAGGGCCAGATCCTGGGG
 F  L  I  G  S  D  P  T  P  Q  K  R  S  L  Q  I  V  T  L  G  N  R  E  G  G  T  W  K  G  Q  I  L  G
GTGCAGGGCGCTGTTACGACAGTTGGAATCACACCGGACACCCAATCTATCGTGGGTTTGTATCGCACCTATGTGGCCATCGCCACTGGCACCGGCATGC
 V  Q  G  A  V  T  T  V  G  I  T  P  D  T  Q  S  I  V  G  L  Y  R  T  Y  V  A  I  A  T  G  T  G  M
AGCGGACCCAGAAGGATCCCAGCACCAACTTCTACCTGTTGTTCAACGCCTGGAGTCCAGATGACGATGCATACTATCCTGATGATGCCGGGCGCTGGGA
Q  R  T  Q  K  D  P  S  T  N  F  Y  L  L  F  N  A  W  S  P  D  D  D  A  Y  Y  P  D  D  A  G  R  W  E
GTACGTCCTCAACTCAAGCGGTCACATCTACCAGGGCGCGGTGGGGTCCGTTAGTGAGCGCTACTGGGTTTACGGACAGTTTGACAAGGGTGTTTTAGAC
 Y  V  L  N  S  S  G  H  I  Y  Q  G  A  V  G  S  V  S  E  R  Y  W  V  Y  G  Q  F  D  K  G  V  L  D
GCCTGCATTTACATCATGGACTCTTGTCAGATGCCTATCCACGACCGAGGGGACATCATCCAAGTTTCCAGAAAAGCATCTGCCATGATGAACTCCCAGG
 A  C  I  Y  I  M  D  S  C  Q  M  P  I  H  D  R  G  D  I  I  Q  V  S  R  K  A  S  A  M  M  N  S  Q
ATGACAACGGTGTGTTGGTTGGGAACTGGAGTGAGGACTTTTCCATGGGAACGCCTCCAACTGCTTGGACTGGCAGCACCAAAATCCTTCAGGATTACTT
D  D  N  G  V  L  V  G  N  W  S  E  D  F  S  M  G  T  P  P  T  A  W  T  G  S  T  K  I  L  Q  D  Y  F
TGGCCAGGGAACCCCAGTCTGCTTTGCCCAGTGTTGGGTGTATGCTGGAGTCCTGTGCTCATTCATGAGAAGCCTGGGCATTCCATGCAGGGTCATCACC
 G  Q  G  T  P  V  C  F  A  Q  C  W  V  Y  A  G  V  L  C  S  F  M  R  S  L  G  I  P  C  R  V  I  T
AATTTCAACTCAGCCCACGACAACACAGGGAACCTGAAGACTGAGCTCATCTTTAAGCCAGACGGCACACCGGACAGACGCAACACCAGGGACTCCATCT
 N  F  N  S  A  H  D  N  T  G  N  L  K  T  E  L  I  F  K  P  D  G  T  P  D  R  R  N  T  R  D  S  I
GGAACTACCACTGCTGGTGTGAAGCCTTCATGAAGCGCTCTGATCTTCCACCAAAGTACGCCGGATGGCAGGTGGTGGACGCCACACCACAGGAGACCAG
W  N  Y  H  C  W  C  E  A  F  M  K  R  S  D  L  P  P  K  Y  A  G  W  Q  V  V  D  A  T  P  Q  E  T  S
CGACGGATATTTTCGATGTGGTCCTGCTCCTGTCATCGCACTCAAGGATGGTGATTTGAACCACCAGTTTGACTGCAGGTTTGCATTTGCTGAGGTGAAC
 D  G  Y  F  R  C  G  P  A  P  V  I  A  L  K  D  G  D  L  N  H  Q  F  D  C  R  F  A  F  A  E  V  N
AGTGACATTGTGTATATAAAGATGGATCGCTATGGCAACATGAACGTCTTTAACACAGACACAACGTCGGTTGGATCGTTAATCCTTACTAAAGCTGTGG
 S  D  I  V  Y  I  K  M  D  R  Y  G  N  M  N  V  F  N  T  D  T  T  S  V  G  S  L  I  L  T  K  A  V
GCACTAATGGGAGTGAGGATATCACACAGAATTACAAGTACCCCGAAGGAAGTCGTGAGAATAGCAATACCATGAGTCAGGCGGAGCAATTTGGCCTGGA
G  T  N  G  S  E  D  I  T  Q  N  Y  K  Y  P  E  G  S  R  E  N  S  N  T  M  S  Q  A  E  Q  F  G  L  E
GAGGGACAACTCTGAAATGCCGGAAACAAAGCTGTCAGCCACAATCACTGTTGACCCGTGCAATCTGGGTGATACTGTGAAGCTGACAGTGACCTTCAAA
 R  D  N  S  E  M  P  E  T  K  L  S  A  T  I  T  V  D  P  C  N  L  G  D  T  V  K  L  T  V  T  F  K
AATCAGAACCAGGTGGACAAAACGATCAAAGCTCACCTGGAAGTGTCTGCTGTTTTCTACACTGGAGTCGTGCTGGATGAATTCAAAGTGGAGGATTTCA
 N  Q  N  Q  V  D  K  T  I  K  A  H  L  E  V  S  A  V  F  Y  T  G  V  V  L  D  E  F  K  V  E  D  F
ACATTTACACAGCAGCTTTTCAGAGCAACTCGGCTGTGTTTGAAATCTTGCCTCAGGAGTACATGCCTCATCTGGGCTCTAAAATGTCTTTGCACTTTAT
N  I  Y  T  A  A  F  Q  S  N  S  A  V  F  E  I  L  P  Q  E  Y  M  P  H  L  G  S  K  M  S  L  H  F  I
CGTCACCGGAAAAACTGAAGACGAGAACGTGTCTGATGTCAAAGTGATCTTTCTGAAGCCCCCTCCTCTCATTGTGCAGCTGAGCGGCCATCCTCAGGTC
 V  T  G  K  T  E  D  E  N  V  S  D  V  K  V  I  F  L  K  P  P  P  L  I  V  Q  L  S  G  H  P  Q  V
AACCAGCAAATGTTTGTGACCGTGTACTTCAAGAACCCCCTCAACATGTCTCTGTACAACGCCAGGCTGGTTATGGAGGGCGCGGCGCTCCTGGACCACA
 N  Q  Q  M  F  V  T  V  Y  F  K  N  P  L  N  M  S  L  Y  N  A  R  L  V  M  E  G  A  A  L  L  D  H
GGGAGTTTAAGTACAGCGTCATCGGTCCAAACATGGAGATCTCCCAGAAGGTGGCCTTCAGACCCCAGAAGCCCGGCTGGAGGAGCATCGTGGCTGTACT
R  E  F  K  Y  S  V  I  G  P  N  M  E  I  S  Q  K  V  A  F  R  P  Q  K  P  G  W  R  S  I  V  A  V  L
TGACTGCCAGAACCTGACGGAGGTCACAGGAAAAGTGGACGTCCAGATCATGCCATGAGCCTCAGATCTTCATCTGCTGCTTCTAGACCATGGAGGAGCT
 D  C  Q  N  L  T  E  V  T  G  K  V  D  V  Q  I  M  P  *
CCTTCACCTCCTCGTGTGATCTTAATGCTTGGAAATGAGCTAAAGACAAATCTGAACTATTGATTGTTTCTTCACAGATCTGGCAGCGCTTTAACTCTTC
GACCAAATCCCTTGGATTAAACTGGATTTAAAGTGTTATGATGTCGTTAGAAGCAGAGTAACTAACGTACCATCTGACCACAGAAGCAGATTGTTATCAG
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Figure 1-3. The nucleotide and deduced amino acid sequences of the full-length *f13a1a* cDNA in medaka.

```
TGCAGTTTATCCTTTACGTTTAGAGACAGCTGGAGGTGTAATGGCAGACAGGCGTACCAGAAGCATGAAGAACTCAAATCTGAGCAGGACCGCAGTGAAC
                                          M  A  D  R  R  T  R  S  M  K  N  S  N  L  S  R  T  A  V  N
CGGGCCTCGATGATTGCTTTACAGCTCCAGGGCGAGTCTTCCAAGTTACTGGAGCTCTATAGACAGAAGGAGAGCTTTCCTGCAGATGCGACTGATGGAC
 R  A  S  M  I  A  L  Q  L  Q  G  E  S  S  K  L  L  E  L  Y  R  Q  K  E  S  F  P  A  D  A  T  D  G
GTCTGGTTTTGGTCCCTCCTCCTTCCTCTCAGCTGGACACCAGAGACAAGCTGTGGCGCCTCCACTCTGCTCTGCTGCAAACCCAAAGCTTGTTGGAGAG
R  L  V  L  V  P  P  P  S  S  Q  L  D  T  R  D  K  L  W  R  L  H  S  A  L  L  Q  T  Q  S  L  L  E  R
GGCCATCACCAAAGAGGAGGAAGAACTGGGTGGTGGGATAAAGGGGGAGTATGAGAACCAGAGGAAGACTGTGAAGGGAAGGCTTTCTTTCCTCCTTCTC
  A  I  T  K  E  E  E  E  L  G  G  G  I  K  G  E  Y  E  N  Q  R  K  T  V  K  G  R  L  S  F  L  L  L
ACCACTGGAAACCTCCTCAAAGCTGTCGATGGAGCTGCCAGTTTGACTCCAAGCCTTGAGGGGTTGGAGTTAGATGGTCCGGCCACTCTGTTTCAGCTGA
 T  T  G  N  L  L  K  A  V  D  G  A  A  S  L  T  P  S  L  E  G  L  E  L  D  G  P  A  T  L  F  Q  L
AGCTATGGCTGCTTCAGGTCTTCAGCGAGGTGCATCACTGGAGCAAGGCAGCCGTGACCACCCTGCAAGAGCTGCTGACGAATGCTGCAGACGAGCGAAG
K  L  W  L  L  Q  V  F  S  E  V  H  H  W  S  K  A  A  V  T  T  L  Q  E  L  L  T  N  A  A  D  E  R  S
CAGCACCACCCGGGTCAGGAGCACAAGAAGTGCTCGACGATGAGAAGGAGACAGGAGGAGGGTGTCTGTGGCAGAAATTACTACGCTCCATGCCAGGTAT
  S  T  T  R  V  R  S  T  R  S  A  R  R  *
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TAACAAACAACAAATAAATCTGACATTTAAAAAATTTCAACCAGAAATAGTCTTCATTTTTTTTAAATCAATTAAAGACAGATAAAGAAAGATATTCTTA
AAAAAAAATGAAATAGCTATTTGATCCATTGATTTAAAGTTGGTCAGCCCTCATTCTCATTGATATCTGTCTGTGTAAATTATGCATTTTTAATGTGGAA
TTTACTGGGTACTCTGGTTGATGTTGGATATTTTTCAGGCAGAAATATTTCAATTGCCTGCAGGAATGACCCTGCTGGATGAAATTCAAAAATATGCACA
GATTACAAGTCACAAACCTTTTTCTTCACATGCAGGGTTTATGATGCGGATAAAGAAACTGGCTTTAGATTGTCGACTGAATGAACTAAGTACCAAATGT
TTGTGTTTGCTCACATAGCAGAGCCAAGTTTGATGTTTTTTTTACTCTTCAGAAGCGCTAAACACCCCTCCCTTTCTTTACTTTGTTTCTTCATTTTTTA
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AATTTACTCCTTCAAAATATTATATTATTTGTTGATTACTGATTGAGCATTTTAATTGCATGTTTGGGGTTTTACCTGTATGGTCATTGGTCTACTTTTG
TATACAAAGCTTGTTGCTGCCGTGGGGACAATATTCTACAGTTTCAGCTTTGAATAAGTTTCGGCTACAGGAGAAGAATCTGTTGCTTCCAAGTGTCATG
TGATCCCAGCTGAACTGAAAAGATATTTAGAATAAATTAACTAGAAATGCAGCCACAAAGAATTTAAACAAAATGTTTAAATTATTTTTTTGCTTACAAG
GGGTCAGATTCCTACAAAGAAATTGATTTTATTTTGAATGGAAGAAGCAGAGATGGCCAATGTAAATTCCATGTTTTGCAAACGATAATTATTGTTTTTT
TTCTTTATTTTATCTTGAAGGGAAGAAGCTGGTGAGGTTTAATGTCAATTCCACGTCATGTTTCATGTTTTGCAAACATTTTTCAAACTATATTGTGTTT
TGTTGTTGTTGTTGGCGGAGGGTCAGCAGTGAAATAAACTGTTTTTGATTATTGTCT(A)n
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Figure 1-4. The nucleotide and deduced amino acid sequences of the full-length *cntfa* cDNA in medaka.

```
ACCATCATGGTGATACGTCCTTGCTGTTCGACAGCAATCGCAGCACTGAGCGGTTCCACAGACAGCTGGGAGATACCCTGCAGGTCATGCCACTTAATGT
                                                                                                  M  P  L  N  V
GGTGTTGGATGGGCCGGCCCCCTGGGGTTTCCGCCTGACAGGAGGAAGGGACTTTAACCAGCCCCTGACCATCTCCAGGATCACTCCAGGCAGTAAGGCG
 V  L  D  G  P  A  P  W  G  F  R  L  T  G  G  R  D  F  N  Q  P  L  T  I  S  R  I  T  P  G  S  K  A
TCCTCAGCCAACTTGTGTCCTGGGGATGTTATCCTGGCCATCGAGGGAGTCCCAGCTACAGACATGCTGCACTGTGAGGCTCAAAACAAGATAAAGGAGT
 S  S  A  N  L  C  P  G  D  V  I  L  A  I  E  G  V  P  A  T  D  M  L  H  C  E  A  Q  N  K  I  K  E
CCAGCAAACAGCTGTGTCTCACTGTGGAAAGGAATCAATCACGACTCTGGTCGCCTCATGTTATGGAGGACGGCAGAGCTCACCCGTTTAAAGTCGACCT
S  S  K  Q  L  C  L  T  V  E  R  N  Q  S  R  L  W  S  P  H  V  M  E  D  G  R  A  H  P  F  K  V  D  L
TGAGACCAAACAGCAGGAGTATAAACCGATCGGCGCCACTCACAACCGCAGAGCTCAGCCGTTTATTGCTGCAGCAAACATTGATGACAAACGACAGGTG
  E  T  K  Q  Q  E  Y  K  P  I  G  A  T  H  N  R  R  A  Q  P  F  I  A  A  A  N  I  D  D  K  R  Q  V
GTCAGCACGTCCTACAACACTCCCATAGGCCTGTACTCTTCAGGAAACATCCAAGATGCCATGGAGGGCCAGATCCGAGGCCTTGTTCAGCCCAAGCCTG
 V  S  T  S  Y  N  T  P  I  G  L  Y  S  S  G  N  I  Q  D  A  M  E  G  Q  I  R  G  L  V  Q  P  K  P
AGAGTCCCAGAGCTCTGAGCAGCATCGAGGAATCTGACGTGTACCGGATGCTGCAGAAAGACCAGGATATGCCCCAGGAGCCTCGGCAGTCTGGCTCCTT
E  S  P  R  A  L  S  S  I  E  E  S  D  V  Y  R  M  L  Q  K  D  Q  D  M  P  Q  E  P  R  Q  S  G  S  F
TAGAGCCCTCCAGGAGTTTATCGACAGCGACGGCACTCGTCCCATTGTGACCCGGACAGTCAAAGCCCCCACCTCCAAACCAGCTCCACCTACAGGAAAC
  R  A  L  Q  E  F  I  D  S  D  G  T  R  P  I  V  T  R  T  V  K  A  P  T  S  K  P  A  P  P  T  G  N
TTGCAGAAACTGCCCGTCTGCGATAAGTGTGGGAATGGGATTGTGGGGACAGTGATTAAAGCTCGGGACAAATACCATCACCCCGGGTGTTTCGCGTGCT
 L  Q  K  L  P  V  C  D  K  C  G  N  G  I  V  G  T  V  I  K  A  R  D  K  Y  H  H  P  G  C  F  A  C
CCGACTGTGATGTCAACCTAAAGCAGAAAGGCTATTTCATTGTGGAAGGGCGTCTGTACTGTGAGAGCCACGCCCGAGCCAGAATGAGACCCCCAGAGGG
S  D  C  D  V  N  L  K  Q  K  G  Y  F  I  V  E  G  R  L  Y  C  E  S  H  A  R  A  R  M  R  P  P  E  G
ACACGACCTCATCACAACATTCCACTCACCTTAGAAGCTTCTCCAAGCACTTTGCCAAACATGTTCAGTGATCTGCAATGTTTGTGCAAGATAATCTTAG
  H  D  L  I  T  T  F  H  S  P  *
CAGAACGAATCATGCTAATGTGATGAATTGGAAATCACACTTCACAGCTATGTCTCATTTAAACATTGCCACATT(A)n
```

Figure 1-5. The nucleotide and deduced amino acid sequences of the full-length *pdlim3a* cDNA in medaka.

```
Medaka F13a1a   MRKAQLSMKG-------------RFQRPVPTSNLHLEDEADYREFEPFERDVGFFPRAPAGDDGSALQVMTIDMQKDQNAGS--HNTVDYINTPL
Medaka F13a1b   MSDTSATPTSPPAPTPTPSKGNRRGRKAGPADN----SNSEFANFPEVEYFIAPGPRGYPP-LTEFLDIWDVDMIRRIDESNKLEHHTELYNSEN
Human F13A1     MSETSRTAFG--------------GRRAVPPNN----SNAAEDDLPTVE-LQGVVPRGVN--LQEFLNVTSVHLFKERWDTNKVDHHTDKYENNK
Mouse F13A1     MSDTPASTFG--------------GRRAVPPNN----SNAAEVDLPTEE-LQGLVPRGVN--LKDYLNVTAVHLFKERWDSNKIDHHTDKYDNNK

Medaka F13a1a   LVVRRGQEFAINITFNRPLAPEDD-VQIEFLIGSDPTPQK-RSLQIVTLGNREGGTWKGQILGVQGAVTTVGITPDTQSIVGLYRTYVAIATGTG
Medaka F13a1b   LIVRRGQEFQIKLTFNRPYKPDEDKFAVEFTIGSSPQFSKGTYIPVFFTKERQSS-WAGRAIANSDNIVTVGITPATNCIVGKYNLYVAVVTPYG
Human F13A1     LIVRRGQSFYVQIDFSRPYDPRRDLFRVEYVIGRYPQENKGTYIPVPIVSELQSGKWGAKIVMREDRSVRLSIQSSPKCIVGKFRMYVAVWTPYG
Mouse F13A1     LIVRRGQTFYIQIDFNRPYDPRKDLFRVEYVIGRYPQENKGTYIPVPVVKELQSGKWGAKVIMNEDRSVRLSVQSSPECIVGKFRMYVAVWTPYG

Medaka F13a1a   MQRTQKDPSTNFYLLFNAWSPDDDAYYPDDAGRWEYVLNSSGHIYQGAVGSVSERYWVYGQFDKGVLDACIYIMDSCQMPIHDRGDIIQVSRKAS
Medaka F13a1b   VRRQRPDKSREMYILFNPWAKDDAVFLDDEMERQECVMNEMGIIYHGAYNDVAERSWNYGQFNYGVLDACLFILDHSEMPIINRGDPVKVTRQAS
Human F13A1     VLRTSRNPETDTYILFNPWCEDDAVYLDNEKEREEYVLNDIGVIFYGEVNDIKTRSWSYGQFEDGILDTCLYVMDRAQMDLSGRGNPIKVSRVGS
Mouse F13A1     ILRTRRDPETDTYILFNPWCEEDAVYLDDEKEREEYVLNDIGVIFYGDFKDIKSRSWSYGQFEDGILDTCLYVMDKAEMDLSGRGNPIKVSRVGS

Medaka F13a1a   AMMNSQDDNGVLVGNWSEDFSMGTPPTAWTGSTKILQDYFGQGTPVCFAQCWVYAGVLCSFMRSLGIPCRVITNFNSAHDNTGNLKTELIFKPDG
Medaka F13a1b   AMMNSQDDNGVLVGDWSGDYIYGVSPTSWTGSTDILISYARSKAPVRYAQCWVYAGVFNTFLRCLGIPSRVVTNFFSAHDNDGNLKTDIILDENG
Human F13A1     AMVNAKDDEGVLVGSWDNIYAYGVPPSAWTGSVDILLEYRSSENPVRYGQCWVFAGVFNTFLRCLGIPARIVTNYFSAHDNDANLQMDIFLEEDG
Mouse F13A1     AMVNAKDDEGVLVGSWDNVYAYGIPPSAWTGSVDILLEYRSSETPVRYGQCWVFAGVFNTFLRCLGIPARVITNYFSAHDNDANLQMDIFLEEDG

Medaka F13a1a   TPDRRNTRDSIWNYHCWCEAFMKRSDLPPKYAGWQVVDATPQETSDGYFRCGPAPVIALKDGDLNHQFDCRFAFAEVNSDIVYIKMDRYGNMNVF
Medaka F13a1b   KIDKNRTRDSVWNYHCWNECYMSRPDLPQGYGGWQIVDATPQETSDGMYRCGPASVHAIKHGEICFPYDAAFVFAEVNSDVVFYSRRRDGTLDLV
Human F13A1     NVNSKLTKDSVWNYHCWNEAWMTRPDLPVGFGGWQAVDSTPQENSDGMYRCGPASVQAIKHGHVCFQFDAPFVFAEVNSDLIYITAKKDGTHVVE
Mouse F13A1     NVSSKLTKDSVWNYHCWNEAWMTRPDLPVGFGGWQAVDSTPQENSDGMYRCGPASVQAVKHGHVCFQFDAPFVFAEVNSDLVYITAKQDGTHVVE

Medaka F13a1a   NTDTTSVGSLILTKAVGTNGSEDITQNYKYPEGSRENSNTMSQAEQFGLER-DNSE---MPETKLSATITVDPCNLGDTVKLTVTFKNQNQVDKT
Medaka F13a1b   KVNRTHIGRMVLTKAPGEETRRDITSQYKFPEGSAEERTVLEKAEEYGCKRVKDNP----SLADVDLTLPTLEISVGDDFELDLEFVNHTKEKRT
Human F13A1     NVDATHIGKLIVTKQIGGDGMMDITDTYKFQEGQEEERLALETALMYGAKKPLNTEGVMKSRSNVDMDFEVENAVLGKDFKLSITFRNNSHNRYT
Mouse F13A1     AVDATHIGKLIVTKQIGGDGMQDITDTYKFQEGQEEERLALETALMYGAKKTLNTEGVVKSRSDVTMNFDVENAVLGKDFKVTITFQNNSSNLYT

Medaka F13a1a   IKAHLEVSAVFYTGVVLDEFKVEDFNIYTAAFQSNSAVFEILPQEYMPHLGSKMSLHFIVTGKTEDEN--VSDVKVIFLKPPPLIVQLSGHPQVN
Medaka F13a1b   VAAYISGSVVYYTGVP-AMSPVQTPHCSNWPQKSVKELVQIESKKYMQHLVEQCNLNFIVTGKIKETGKIVTATKLVTLHNPKLTVEVSGNAKVN
Human F13A1     ITAYLSANITFYTGVPKAEFKKETFDVTLEPLSFKKEAVLIQAGEYMGQLLEQASLHFFVTARINETRDVLAKQKSTVLTIPEIIIKVRGTQVVG
Mouse F13A1     ILAYLSGNITFYTGVSKKEFKKESFEETLDPFSSKKKEVLVRAGEYMSHLLEQGFLHFFVTARINESRDVLAKQKSIILTIPKITIKVRGAAMVG

Medaka F13a1a   QQMFVTVYFKNPLNMSLYNARLVMEGAALLDHREFKYSVIGPNMEISQKVAFRPQKPGWRSIVAVLDCQNLTEVTGKVDVQIMP----
Medaka F13a1b   EEMMVTVEFTNPFSFSLEDVNVRMEGPGVMLPRRKTFSLILGGSHLTWTELFSPQRPGKTRIIATLDCPSLRQVDGQVSLTVEP----
Human F13A1     SDMTVTVEFTNPLKETLRNVWVHLDGPGVTRPMKKMFREIRPNSTVQWEEVCRPWVSGHRKLIASMSSDSLRHVYGELDVQIQRRPSM
Mouse F13A1     SDMVVTVEFTNPLKETLQNVWIHLDGPGVMRPKRKVFREIRPNTTVQWEEVCRPWVSGHRKLIASMTSDSLRHVYGELDLQIQRRPTM
```

Figure 1-6. Alignment of deduced amino acid sequences of F13a1a in medaka and other representative F13A1/F13a1 proteins. Identical amino acids are shown in white letters on a black background.

```
Medaka Cntfa          MADRRTRSMK----NSNLSRTAVNRASMIALQLQGESSKLLELYRQKESFPADATDG--RLVLVPPPS--SQLDTRDKLWRLHSALLQ
Rainbow trout Cntfb   MADQEHIEIDTLLDMPAPGRSRTGRAAALARLLHQDCTYLLELYRERESLLSDHTPAGDRIVSLSLSS--PDLSSDEQVQLLHSALRK
Human CNTF            MAFTEHSPLT------PHRRDLCSRSIWLARKIRSDLTALTESYVKHQGLNKNINLDSADGMPVASTDQWSELTEAERLQENLQAYRT
Mouse CNTF            MAFAEQSPLT------LHRRDLCSRSIWLARKIRSDLTALMESYVKHQGLNKNISLDSVDGVPVASTDRWSEMTEAERLQENLQAYRT

Medaka Cntfa          TQSLLERAITKEEEELGGGIKGEYENQRKTVKGRLSFLLLTTGNLLKAVDGAASLTPSLEGLELDGPATLFQLKLWLLQVFSEVHHWS
Rainbow trout Cntfb   CLGLLECLILREEEEMG-ELEGEYETVRKGVRDRLGHLLHSTKVLLETEEDVTPDHQCNE--EVDGVVGTFGAKMWTYRVLLELIHWA
Human CNTF            FHVLLARLLEDQQVHFT-PTEGDFHQAIHTLLLQVAAFAYQIEELMILLEYKIPRNEADGMPINVGDGGLFEKKLWGLKVLQELSQWT
Mouse CNTF            FQGMLTKLLEDQRVHFT-PTEGDFHQAIHTLTLQVSAFAYQLEELMALLEQKVPEKEADGMPVTIGDGGLFEKKLWGLKVLQELSQWT

Medaka Cntfa          KAAVTTLQELLTNAADERSSTTRVRSTRSARR
Rainbow trout Cntfb   NSASQTLHVLHS----EREGREEI--------
Human CNTF            VRSIHDLRFISSHQTGIPARGSHYIANNKKM-
Mouse CNTF            VRSIHDLRVISSHHMGISAHESHYGA--KQM-
```

Figure 1-7. Alignment of deduced amino acid sequences of Cntfa in medaka and other representative CNTF/Cntf proteins. Identical amino acids are shown in white letters on a black background.

```
Medaka Pdlim3a      MPLNVVLDGPAPWGFRLTGGRDFNQPLTISRITPGSKASSANLCPGDVILAIEGVPATDMLHCEAQNKIKESSKQLCLTVERNQSRLWSP
Zebrafish Pdlim3a   MPQNVVLDGPAPWGFRLSGGKDFNQPLTITRVTPGSKASRVNLCPGDIILSIQGVSTDGMTHAEAQNNIKDSTNQLFLKIERPETKIWSP
Human PDLIM3        MPQTVILPGPAPWGFRLSGGIDFNQPLVITRITPGSKAAAANLCPGDVILAIDGFGTESMTHADAQDRIKAAAHQLCLKIDRGETHLWSP
Mouse PDLIM3        MPQNVVLPGPAPWGFRLSGGIDFNQPLVITRITPGSKAAAANLCPGDVILAIDGFGTESMTHADAQDRIKAASYQLCLKIDRAETRLWSP

Medaka Pdlim3a      HVMEDGRAHPFKVDLETKQQEYKPIGATHNRRAQPFIAAANIDDKRQVVSTSYNTPIGLYSSGNIQDAMEGQIRGLVQPKPESPRALSSI
Zebrafish Pdlim3a   QVIEEGKVNPFKINLEAEKQEYKPIGTGHNRRAQPFVAAASLDDKTQVVSSTYNTPIGLYSQDNIQDALQGQIRGLVHEKPEGSKPLTSI
Human PDLIM3        QVSEDGKAHPFKINLESEPQEFKPIGTAHNRRAQPFVAAANIDDKRQVVSASYNSPIGLYSTSNIQDALHGQLRGLIPSSPQNEPTASVP
Mouse PDLIM3        QVSEDGKAHPFKINLEAEPQEFKPIGTAHNRRAQPFVAAANIDDKRQVVSASYNSPIGLYSTSNIQDALHGQLRGLIPGSLQNEPTASVP

Medaka Pdlim3a      EESDVYRMLQKDQDMPQEPRQSGSFRALQEFIDS-DGTRPIVTRTVKAPTSKPAPPTGNLQKLPVCDKCGNGIVGTVIKARDKYHHPGCF
Zebrafish Pdlim3a   EDSHVYRMLQDANEQPHEPRQSGSFKALQDYVES-DGTRPMVTRSVKAPVTKPQAATSSLQKLPLCDKCGTGIVGTVVKARDKYRHPACF
Human PDLIM3        PESDVYRMLHDNRNEPTQPRQSGSFRVLQGMVDDGSDDRPAGTRSVRAPVTKVHGGSGGAQRMPLCDKCGSGIVGAVVKARDKYRHPECF
Mouse PDLIM3        PQSDVYRMLHDNRDDPAAPRQSGSFRVLQDLVNDGPDDRPAGTRSVRAPVTKVHGGAGSAQRMPLCDKCGSGIVGAVVKARDKYRHPECF

Medaka Pdlim3a      ACSDCDVNLKQKGYFIVEGRLYCESHARARMRPPEGHDLITTFHSP
Zebrafish Pdlim3a   VCSDCGMNLKQKGYFFVDGQMYCEAHARMRMTPPEGHDLVTVYPTA
Human PDLIM3        VCADCNLNLKQKGYFFIEGELYCETHARARTKPPEGYDTVTLYPKA
Mouse PDLIM3        VCADCNLNLKQKGYFFVEGELYCETHARARTRPPEGYDTVTLYPKA
```

Figure 1-8. Alignment of deduced amino acid sequences of Pdlim3a in medaka and other representative PDLIM3/Pdlim3 proteins. Identical amino acids are shown in white letters on a black background.

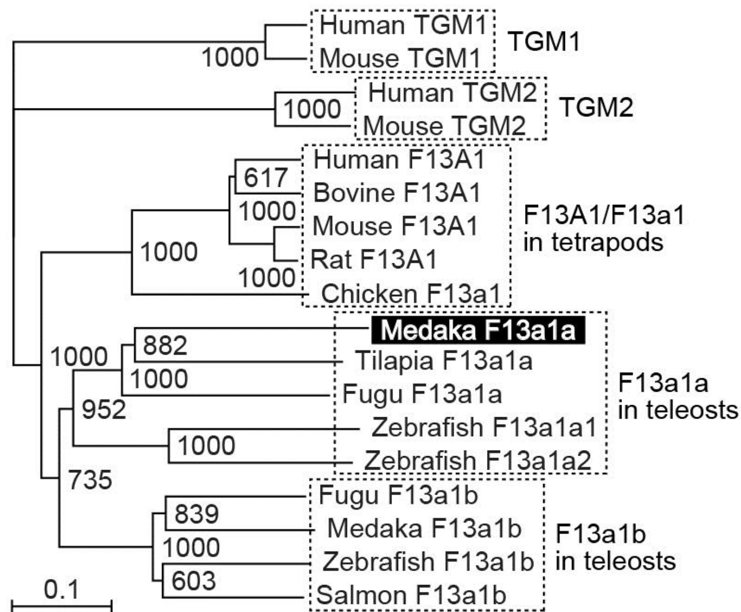

Figure 1-9. Phylogenetic analyses of F13A1/F13a1. The number at each node indicates bootstrap values for 1000 replicates. Scale bars represent 0.1 substitutions per site. Note that because this analysis revealed the presence of two paralogs for F13a1 in the teleost lineage, they were designated F13a1a/F13a1b (the product of the medaka transcript that was identified in this thesis were designated F13a1a).

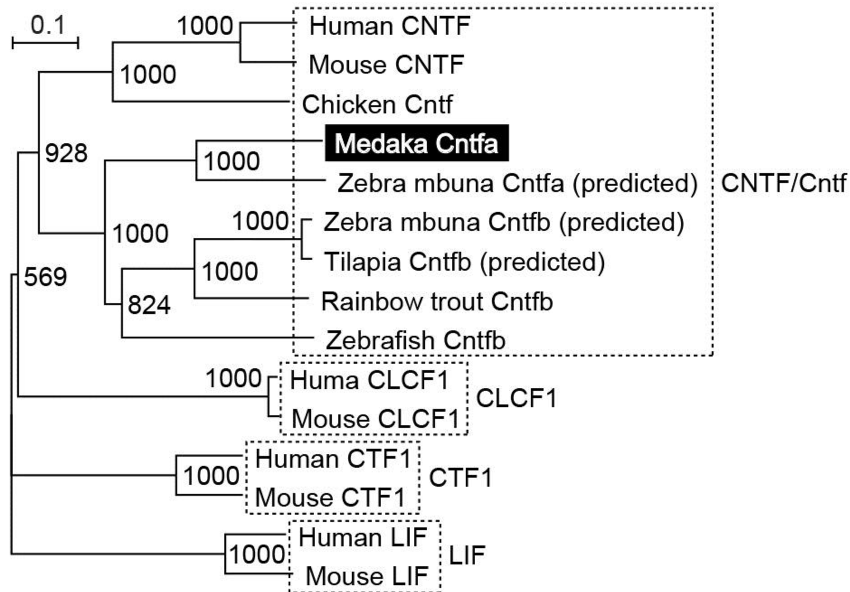

Figure 1-10. Phylogenetic analyses of CNTF/Cntf. The number at each node indicates bootstrap values for 1000 replicates. Scale bars represent 0.1 substitutions per site. Note that because this analysis revealed the presence of two paralogs for Cntf in the teleost lineage, they were designated Cntfa/Cntfb (the product of the medaka transcript that was identified in this thesis were designated Cntfa).

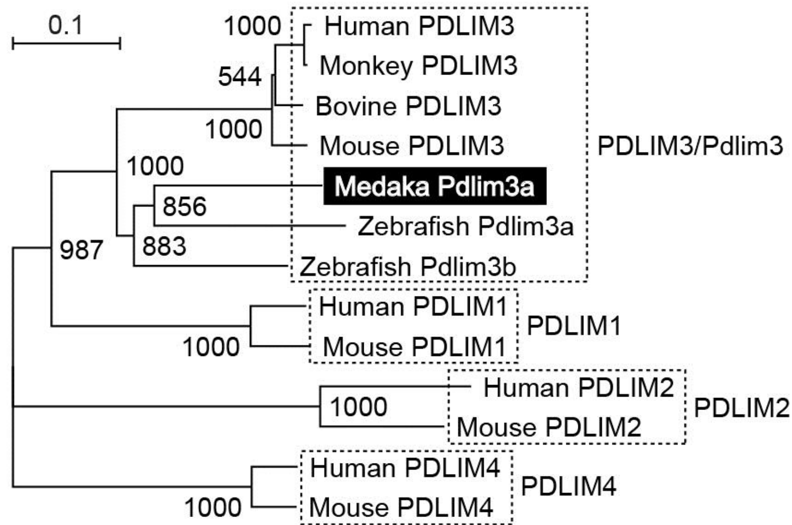

Figure 1-11. Phylogenetic analyses of PDLIM3/Pdlim3. The number at each node indicates bootstrap values for 1000 replicates. Scale bars represent 0.1 substitutions per site. Note that because this analysis revealed the presence of two paralogs for Pdlim3 in the teleost lineage, they were designated Pdlim3a/Pdlim3b (the product of the medaka transcript that was identified in this thesis were designated Pdlim3a).

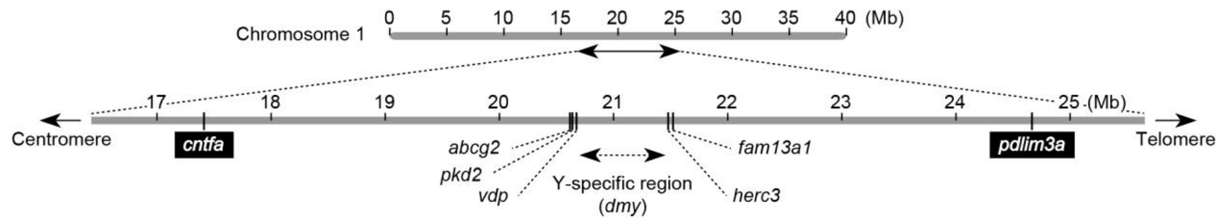

Figure 1-12. Location of *cntfa* and *pdlim3a* on the medaka sex chromosome (chromosome 1). The medaka sex chromosome is 40 Mb in length. Note that the X and Y chromosomes in medaka are genetically the same, with the exception that the Y chromosome has an additional 258-kb sequence (Y-specific region) that includes the sex-determining gene *dmy* (Kondo *et al*., 2006). The Y-specific region has not been precisely mapped, but it is known to lie between *abcg2*/*pkd2*/*vdp* and *herc3*/*fam13a1*.

Chapter 2

Unraveling the mechanisms underlying female-biased expression of *f13a1a* in the medaka brain

Introduction

In Chapter 1, I identified *f13a1a*, a member of the transglutaminase family genes, as exhibiting female-biased expression in the medaka brain. Quantitative evaluation showed that *f13a1a* was 16.1-fold more highly expressed in the female brain than in the male brain. This is the largest sex difference ever reported for any gene in any species (except sex chromosome-linked genes that are present and expressed only in one sex), suggesting that *f13a1a* may play some role in sexual differentiation of the brain. A question then arises as to the mechanisms underlying this very large sex difference in *f13a1a* expression. Despite the fundamental biological roles of F13A1, little is known about the regulatory mechanisms for its expression; in particular, no information is available for the expression in the brain.

In this Chapter, I examined the detailed expression pattern and regulatory mechanisms of *f13a1a* in the medaka brain. Results demonstrated that *f13a1a* was female-specifically expressed in neurons located in the preoptic area and female-predominantly expressed in cells lying scattered immediately outside the brain parenchyma. It is known that sex steroid hormones synthesized and released from the gonad affect sexual differentiation of the brain (Phoenix *et al*., 1959; McDonald *et al*., 1970; MacLusky and Naftolin, 1981). In addition, emerging evidence indicates that some parts of the brain sexually differentiate according to a cell-autonomous genetic program governed by sex chromosome complement (Carruth *et al*., 2002; de Vries *et al*., 2002; Agate *et al*., 2003). Accordingly, here I assessed both hormonal and genetic influences on *f13a1a* expression, and found that its female-biased expression in the brain resulted from the transient and reversible action of estrogen secreted by the ovary. More importantly, expression of *f13a1a* could be induced even in sexually mature males by treatment with estrogen, thereby being completely sexually reversible even in adulthood. These data imply that *f13a1a* may possibly contribute to the development and reversal of sex

differences in the brain and some physiological/behavioral traits.

Materials and Methods

Animals

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Tokyo. Medaka of the d-rR strain were maintained at 28°C with a 14-hour light/10-hour dark photoperiod and were fed 3 or 4 times per day with live brine shrimp and commercial pellet food (Otohime; Marubeni Nisshin Feed). Sexually mature adults of 3–5 months of age were sampled at 0.5–3 hours following the onset of light and used for analyses unless otherwise noted.

Spatial analysis of *f13a1a* expression

Sex differences in the spatial expression patterns of *f13a1a* in the brain were examined by real-time PCR. The whole brain was removed from male and female medaka (n = 6 for each sex) and divided into 3 portions: (i) the olfactory bulb, telencephalon, diencephalon, and mesencephalon except the optic tectum (OB/Tel/Die/Mes); (ii) the optic tectum (OT); and (iii) the cerebellum and medulla oblongata (Cb/MO). Total RNA was isolated separately from each portion using the RNeasy Plus Universal Mini Kit (Qiagen), and cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). PCR was performed using LightCycler 480 SYBR Green I Master on a LightCycler 480 System II (Roche). A melting curve analysis was conducted for every reaction to ensure that a single amplicon was produced in each sample. The expression of *actb* in each sample was used to normalize the expression of target transcripts by the standard curve method. The primers used for real-time PCR are listed in Table 2-1.

More detailed analysis of spatial expression was performed by *in situ* hybridization. The DNA fragment corresponding to nucleotides 1,789–2,837 of the medaka *f13a1a* cDNA was PCR-amplified using the primers listed in Table 2-1 and served to generate digoxigenin (DIG)-labeled cRNA probes using the DIG RNA Labeling Mix (Roche Diagnostics). The whole brain dissected from male and female medaka (n = 5 for each sex) was fixed in 4%

paraformaldehyde (PFA) for 7–8 hours, dehydrated in ethanol, and embedded in paraffin. Serial coronal sections of 10 μm thickness were cut from the olfactory bulb throughout the medulla oblongata. The sections were digested with proteinase K (Wako Pure Chemical Industries, Osaka, Japan) for 15 min at 37°C, postfixed with 4% PFA for 10 min, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 15 min. Hybridization was conducted with the above-described DIG-labeled probes in hybridization buffer (50% formamide, 5× saline-sodium citrate (SSC), 5× Denhardt's solution, 2 mg/ml yeast RNA, and 30 μg/ml calf thymus DNA) overnight at 55°C. The sections were washed in 5× SSC, 50% formamide for 20 min at 55°C and in 2× SSC for 2 × 20 min at 55°C. The hybridized probes were visualized using alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche Diagnostics) in a dilution of 1:2000 and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Roche Diagnostics), following the manufacturer's instructions. The colour was allowed to develop for 7 hours in the dark.

The subdivisions and nomenclature of brain nuclei were taken from the medaka brain atlases (Anken and Bourrat, 1998; Ishikawa *et al*., 1999; http://www.shigen.nig.ac.jp/medaka/medaka_atlas/).

Temporal analysis of *f13a1a* expression

The expression profiles of *f13a1a* during growth and sexual maturation were assessed by real-time PCR using the whole brain of male and female medaka (n = 8 for each sex) at the following stages: 1 month of age, at which stage secondary sexual characteristics begin to appear; 2 months of age, at which stage fish are juvenile and have not yet spawned; 3 months of age, at which stage fish have become sexually mature and spawned; and 7 months of age, at which stage fish have regressed somewhat and the frequency of spawning has declined. Total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen) and reverse-transcribed to cDNA using the Omniscript RT Kit (Qiagen). Real-time PCR was performed as described above.

Evaluation of genetic and phenotypic sex dependence of *f13a1a* expression

Next, I investigated whether sex differences in *f13a1a* expression coincided with the

genetic sex and/or phenotypic sex by producing and examining sex-reversed medaka. XX gonadal males and XY gonadal females were produced as described previously (Okubo *et al.*, 2011). Briefly, fertilized eggs were incubated at high temperature (32°C) and simultaneously received 0.2 ng/ml methyltestosterone until hatching, which led to the production of XX gonadal males. XY gonadal female fish were obtained by exposing fertilized eggs to 200 ng/ml 17β-estradiol (E2) until hatching. The whole brain was removed from these sex-reversed XX males and XY females and wild-type XY males and XX females (n = 8 for each group). Total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen) and reverse-transcribed to cDNA using the Omniscript RT Kit (Qiagen). Real-time PCR was performed as described above.

Evaluation of hormonal effects on *f13a1a* expression

The ovary was surgically removed from female medaka following the procedure described elsewhere (Kanda *et al.*, 2008). Ovariectomized fish were immersed in water containing 100 ng/ml of E2 or 11-ketotestosterone (11KT; the most prominent, non-aromatizable teleost androgen) or the vehicle alone (0.001% ethanol) (n = 8 for each group) for 5 days. The steroid concentration used was determined according to previous reports of serum steroid levels in medaka (Foran *et al.*, 2002; 2004; Tilton *et al.*, 2003). Sham-operated female fish (n = 8) treated with the vehicle alone were used as controls. Total RNA was isolated from the whole brain of these fish using the RNeasy Plus Universal Mini Kit (Qiagen), and reverse-transcribed to cDNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). Real-time PCR was carried out to address *f13a1a* expression as described above.

Whole brain of the sham-operated fish, ovariectomized fish, and ovariectomized fish treated with E2 (n = 4 for each group) was also subjected to *in situ* hybridization to examined *f13a1a* expression following the procedure described above, except that color development was allowed to proceed for 10 hours.

Next, a similar series of analyses were conducted for males. The testis was removed from male medaka, which were then treated with E2 (n = 6), 11KT (n = 9), or the vehicle alone (n = 8) as described above for 6 days. Sham-operated male fish (n = 8) were treated with the

vehicle alone as controls. The expression of *f13a1a* in the brain of these fish was assessed by real-time PCR as described above.

In addition, male fish with intact testis were treated with E2 or the vehicle alone following the above-described procedure (n = 4 for each group) for 5 days. The whole brain was dissected from these fish and subjected to *in situ* hybridization for *f13a1a* as described above.

Examination of estrogen receptor (ER) expression in *f13a1a*-expressing neurons

The analysis described above led to the identification of female-specific *f13a1a*-expressing neurons in two brain nuclei: the parvocellular portion of the magnocellular preoptic nucleus (PMp) and the magnocellular portion of the magnocellular preoptic nucleus (PMm). Teleost fish, including medaka, has three paralogous genes for ER, *esr1*, *esr2a*, or *esr2b* (Hawkins *et al*., 2000). I tested the possibility that *f13a1a*-expressing neurons in PMp and PMm also expressed *esr1* and/or *esr2b*, which are suggested to be intimately involved in feminization of the medaka brain (Hiraki *et al*., 2012), by performing double *in situ* hybridization as described earlier (Takeuchi and Okubo, 2013). Briefly, the brain dissected from female medaka was fixed in 4% PFA and embedded in 5% agarose (Type IX-A; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% sucrose. Frozen coronal sections of 20 μm thickness were cut and hybridized with the *f13a1a* probe described above, which was here labeled with fluorescein using the Fluorescein RNA Labeling Mix and T7 RNA polymerase (Roche Diagnostics), and with the DIG-labeled ER (*esr1* or *esr2b*) probe, which was described previously (Hiraki *et al*., 2012). After washing, the sections were reacted with an anti-fluorescein antibody conjugated to horseradish peroxidase (HRP) (PerkinElmer, Waltham, MA, USA) and an anti-DIG antibody conjugated to alkaline phosphatase (AP) (Roche Diagnostics). The anti-fluorescein and anti-DIG antibodies were visualized by the TSA Plus Fluorescein System (PerkinElmer) and Fast Red (Roche Diagnostics), respectively. Fluorescent images were acquired using a confocal laser scanning microscope (C1; Nikon, Tokyo, Japan). The excitation and emission wavelengths for detection were as follows: fluorescein, 488 nm and 515/30 nm; Fast Red, 543 nm and 605/75 nm.

Genomic cloning and sequence analysis of the *f13a1a* locus

The genomic sequence of the medaka *f13a1a* locus was obtained by a BLAST search of the Ensembl genome database (http://www.ensembl.org/Multi/blastview) using the *f13a1a* cDNA as the query sequence. To search for possible estrogen-responsive elements (EREs) in the *f13a1a* locus, the sequence obtained was analyzed by Dragon ERE Finder (version 3; http://datam.i2r.a-star.edu.sg/ereV3/).

The sequence of the locus encoding F13a1a in other teleost species, including tilapia, fugu, and zebrafish, which were used for phylogenetic analysis in Chapter 1, was also obtained and analyzed in the same way.

Statistical analysis

All quantitative data are presented as the mean and the standard error of the mean. In real-time PCR analyses, the expression level of target transcripts (normalized to that of *actb*) in the whole brain of sexually mature adult males was arbitrarily set to 1, and the relative difference was calculated in order to facilitate comparisons among analyses.

Statistical analyses were performed using the GraphPad Prism software (GraphPad Software). Comparisons between two groups of data were evaluated for statistical significance by the unpaired two-tailed Student's *t*-test. When the F-test indicated a significant difference in variance between groups, Welch's correction was applied. Comparisons between more than two groups were evaluated by one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test. In cases where the variances were significantly different among groups, data were analyzed by the non-parametric Kruskal-Wallis test followed by Dunn's *post hoc* test.

Results

Sex differences in the spatial and temporal expression of *f13a1a* in the medaka brain

The expression level of *f13a1a* in three portions of the brain was examined by real-time

PCR and compared between the sexes (Figure 2-1). *f13a1a* was much more highly expressed in females than in males in all brain portions (24.0-fold in OB/Tel/Die/Mes, 90.8-fold in OT, and 877-fold in Cb/MO).

In situ hybridization was conducted to assess sex differences in *f13a1a* expression at the cellular level (Figure 2-2). Neurons expressing *f13a1a* were present in two preoptic nuclei, PMp and PMm, and were found exclusively in females. In addition to these neurons, the expression of *f13a1a* was observed in cells lying scattered along the surface of the brain parenchyma, which were much more numerous in females than in males.

Subsequently, developmental changes in *f13a1a* expression in the brain were examined by real-time PCR to determine when, during growth and sexual maturation, *f13a1a* expression was sexually differentiated (Figure 2-3). The female brain began to exhibit significantly higher *f13a1a* expression than the male brain as early as 2 months of age and this female bias persisted thereafter.

Genetic and phenotypic sex dependence of *f13a1a* expression

In order to define the mechanisms underlying the female-biased expression of *f13a1a*, I first investigated whether the sex difference in *f13a1a* expression coincided with genetic sex or phenotypic sex. The expression level of *f13a1a* in the brain of sex-reversed XX gonadal males and XY gonadal females as well as wild-type XY males and XX females was examined by real-time PCR (Figure 2-4). XY gonadal females exhibited the same high level of *f13a1a* expression as wild-type XX females, whereas XX gonadal males expressed *f13a1a* at a lower level, which was comparable with that of wild-type XY males.

Effects of sex steroid hormones on *f13a1a* expression

The effects of sex steroid hormones on *f13a1a* expression in the female brain were investigated by means of ovariectomy followed by hormone treatment (Figures 2-5 and 2-6). Real-time PCR analysis revealed that ovariectomy caused a substantial decrease in the overall expression of *f13a1a* in the female brain, and this effect was completely reversed by treatment with E2; 11KT had no such effects. *In situ* hybridization showed that the female-specific *f13a1a* expression in the PMp and PMm neurons was abolished by ovariectomy and restored

by treatment with E2. Similarly, the number of *f13a1a*-expressing cells scattered along the brain surface were markedly decreased by ovariectomy and reinstated by E2 treatment.

The effects of sex steroid hormones on *f13a1a* expression in the male brain were also examined (Figures 2-7 and 2-8). Real-time PCR analysis revealed that the overall expression of *f13a1a* in the male brain showed no significant response to castration. Subsequent treatment with E2, however, led to a large increase in the expression, whereas 11KT had no such effects. *In situ* hybridization showed that *f13a1a*-expressing neurons emerged in PMp of the male brain after treatment with E2 (while no *f13a1a*-expressing neurons could be detected in PMm). *f13a1a*-expressing cells scattered along the brain surface were also greatly increased by E2 treatment.

Coexpression of *f13a1a* and ER in the PMp and PMm neurons

I then examined the possible coexpression of *f13a1a* and ER (*esr1* and/or *esr2b*) in the same neurons in PMp and PMm by double *in situ* hybridization (Figure 2-9). In both nuclei, virtually all *f13a1a*-expressing neurons also expressed both *esr1* and *esr2b* (the expression of *esr1* and *esr2b* was detected not only in *f13a1a* neurons but also in other neurons, especially in PMp).

Structural features of the *f13a1a* locus

The medaka *f13a1a* was found to consist of 18 exons and 17 introns, and the first methionine codon was located in exon 2. The search for possible EREs within the 5′ proximal region of *f13a1a* identified two palindromic ERE-like sequences, CGGTCAgagCGTCCT and GGGTCAgggTCACCT, at 591 and 326 bp, respectively, upstream of the *f13a1a* transcription start site (Figure 2-10).

Palindromic ERE-like sequences were also identified in the 5′ proximal region of the predicted *f13a1a* genes in the genome of other teleost species: AGGTCAgcgTGACCT at 215 bp upstream of the transcription start site of the predicted tilapia *f13a1a*; AGGTCAttgTGACCT and TGGACActcTGACCT at 1779 and 1959 bp, respectively, upstream of the first methionine codon of the predicted fugu *f13a1a* (the transcription start site has not been predicted); ATGTCAgacTGACCT at 111 bp upstream of the transcription

start site of the predicted zebrafish *f13a1a2* (no ERE-like sequences were found for *f13a1a1*, the other paralogous gene in zebrafish).

Discussion

In Chapter 1, I identified *f13a1a* as exhibiting highly female-biased expression in the medaka brain. Here, I found that neurons expressing *f13a1a* were present exclusively within two preoptic nuclei, PMp and PMm, and confined to females only. This study is, to the best of my knowledge, the first to demonstrate the neuronal distribution and sex difference in the expression of F13A1/F13a1. It would be worthwhile to ascertain whether or not these two features of F13A1/F13a1 expression are conserved across species. Interestingly, the phenotype of *F13a1*-deficient mice is in part sex-specific, with only males exhibiting cardiac impairment in adulthood (Souri *et al.*, 2008); there is no obvious explanation for this sex difference. Some sex differences, though male-biased, as opposed to the situation in medaka, may also exist in the F13A1 system of mice.

In addition to the preoptic neurons, *f13a1a* is expressed in cells lying scattered immediately outside the brain parenchyma, which are much more numerous in females than in males. It is not known at present what type of cells these are. However, taking into account that F13a1 immunoreactivity has been observed in microglial cells in the optic nerve of goldfish (Sugitani *et al.*, 2012), it seems reasonable to speculate that the *f13a1a*-expressing cells represent microglial cells. There is a need to compare the expression of *f13a1a* and microglial markers such as ionized calcium-binding adapter molecule 1 (*iba1*) and integrin-αM (*itgam*; also called complement component 3 receptor 3 subunit).

To my knowledge, *f13a1a* shows the largest sex difference in expression in the brain ever reported for any autosomal genes. This fact led me to a consideration of the mechanisms responsible for this large sex difference. One clue was the appearance of the sex difference at 2 months of age, suggesting that the sexually dimorphic expression of *f13a1a* is not innate, but rather is established during growth and/or sexual maturation. In line with this, I also found, by analyzing sex-reversed fish whose genetic and phenotypic sex were discordant, that the expression levels of *f13a1a* in the brain were correlated not with genetic sex but rather with

phenotypic sex. These results indicate that the sex difference in *f13a1a* expression is not caused by sex chromosome complement, but relies on hormonal factor(s).

A subsequent series of analyses evaluating the effects of gonadectomy and sex steroid hormone replacement indeed demonstrated that the female-biased expression of *f13a1a* in the brain depends largely, or even solely, on estrogen produced by the ovary. There are two different modes of action of sex steroid hormones in generating sex differences in the brain: either permanent/irreversible or transient/reversible (Arnold, 2009b; McCarthy and Arnold, 2011). My results showed that, in females, removal of circulating estrogen by ovariectomy totally abolished *f13a1a* expression in the PMp and PMm neurons and diminished *f13a1a* expression in cells along the brain surface, which was exactly comparable to the situation in males. Moreover, in males, estrogen administration induced *f13a1a* expression in these neurons and cells to a degree comparable to that seen in females. These findings demonstrate that the sexual phenotype of *f13a1a* expression in the brain is labile, depending on estrogen status, even in adulthood and that the effects of estrogen in this regard are transient and completely reversible. This is of interest when considering that the brain of teleost fish exhibits a considerable degree of sexual plasticity even in adulthood (Godwin, 2010; Munakata and Kobayashi, 2010; Le Page *et al.*, 2010). In the brain of mice and rats, sexually differentiated expression of several genes/proteins possibly underlying sex differences in physiological and behavioral traits is attributed to the permanent and irreversible action of sex steroid hormones. These include, for example, galanin, cholecystokinin, substance P, vasopressin, ER, and progestin receptor (Polston and Simerly, 2003; Westberry *et al.*, 2010; Schwarz *et al.*, 2010; Auger *et al.*, 2011). Such hormonal action should cause the brain to sexually differentiate in a permanent and irreversible manner, which is exactly what happens in the rodent brain. The sexual dimorphism in *f13a1a* expression in the medaka brain, which is readily reversible as oppose to these genes/proteins, may possibly contribute to the lifelong sexual plasticity of the teleost brain.

In addition, I found that virtually all *f13a1a*-expressing neurons in PMp and PMm also express *esr1* and *esr2b*, suggesting that estrogen may directly stimulate the expression of *f13a1a* in these neurons. This possibility is supported by another finding that palindromic ERE-like sequences are present in the proximal promoter region of *f13a1a*.

Importantly, I identified palindromic ERE-like sequences *in silico* in the *f13a1a* proximal promoter region of tilapia, fugu, and zebrafish as well. This strongly suggests that the stimulation of *f13a1a* expression by estrogen, which certainly leads to its female bias, is a conserved phenomenon across teleost species. If this is true, it seems plausible that *f13a1a* plays a general and important role in the process of feminization and/or demasculinization of the teleost brain. Future studies are required to determine whether the female-biased expression of *f13a1a* in the brain is conserved among teleost and what specific role *f13a1a* plays within the brain.

Clearly, the question left to be answered is what is the significance of the sexually dimorphic expression of *f13a1a* in the brain. PMp and PMm, the preoptic nuclei where *f13a1a* is female-specifically expressed, have been implicated, in teleosts, in controlling pituitary hormone secretion and sexual behavior, respectively. The estrogen-dependent sexual dimorphism in *f13a1a* expression in these nuclei may possibly mediate estrogen-induced sex differences in these processes. As no information is available about the role of F13A1/F13a1 in neurons, it is difficult to predict the specific contribution of its sexually dimorphic expression. In contrast to F13A1/F13a1, evidence has accumulated that another member of the transglutaminase family, transglutaminase 2 (TG2), is expressed in neurons and involved in neurite outgrowth and neuronal differentiation and cell death (Ruan and Johnson, 2007). Evidence is also accumulating that TG2 contributes to the pathogenesis of several neurodegenerative disorders, including Huntington's, Alzheimer's, and Parkinson's diseases, by facilitating the formation of soluble oligomers of proteins related to these diseases (Ruan and Johnson, 2007; Iismaa *et al*., 2009; Jeitner *et al*., 2009). In addition, a recent study has demonstrated that TG2 simultaneously alters the expression status of many genes by modifying chromatin structure through N-terminal polyamidation of histone H3 in a model of Huntington's disease and that TG2 inhibition protects striatal neurons from excitotoxicity (McConoughey *et al*., 2010). It can be speculated that F13a1 plays roles similar to those of TG2 in neurons. Future studies are needed to determine the specific substrates of F13A1/F13a1 and its physiological role in neurons.

It may be of interest to note that cross-linking of polyamines to vasoactive intestinal peptide (VIP) by transglutaminase has been shown to alter the biochemical properties of this

neuropeptide, including the affinity to the receptors and susceptibility to proteolytic attack by trypsin (De Maria *et al*., 2002; Caraglia *et al*., 2006). In addition, TG2 has been shown to suppress the expression of *Pac1*, a gene encoding a receptor for another neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP), by cross-linking and inactivating the transcription factor Sp1 (Miura *et al*., 2013). Peptidergic neurons in PMp and PMm produce and respond to a variety of neuropeptides involved in pituitary hormone secretion and sexual behavior. The female-specific expression of *f13a1a* in these nuclei may possibly lead to post-translational modification of neuropeptides and translational modification of their receptors, thereby contributing to sex differences in these processes.

Alternatively, considering recent evidence that the overexpression of F13a1 in retinal ganglion cells promoted neurite outgrowth in these cells in goldfish (Sugitani *et al*., 2012), *f13a1a* may serve to form sex differences in cell morphologies. This idea is supported by preliminary data from our laboratory that show that in medaka, females have much larger PMm neurons than males.

It is also difficult to predict the significance of the female-predominant expression of *f13a1a* in cells along the brain surface, which may represent microglial cells as mentioned above. A recent study has provided evidence that neonatal male mice have more numerous and more activated microglia than females due to the influence of estrogen and that microglia mediate the process through which estrogen induces the masculinization of neuronal morphology and copulatory behavior (Lenz *et al*., 2013). Microglial expression of F13A1/F13a1 may mediate this process, although possibly having opposite effects on sexual differentiation (*i.e.*, masculinization or feminization) of the brain in medaka and mice.

In summary, this chapter clearly demonstrated that estrogen derived from the ovary causes *f13a1a* to be more highly expressed in the female than the male brain. Also shown was that this sex difference is fully reversible even in adulthood, depending on the estrogenic milieu. In addition, evidence was obtained to suggest that the estrogen-dependent female bias of *f13a1a* expression is likely a general property of teleosts. These findings allow us to assume that the sexually dimorphism of *f13a1a* expression may constitute the molecular and neural basis of persistent sexual plasticity of some brain function in teleosts.

Table 2-1. Primers used in Chapter 2.

Target	Direction	Purpose	Sequence (5′ to 3′)
f13a1a	Forward	Real-time PCR	CCTGGACCACAGGGAGTTTAAG
f13a1a	Reverse	Real-time PCR	AGTACAGCCACGATGCTCCTC
actb	Forward	Real-time PCR	GCCTCAAAGCTATTATTTTACTCAACT
actb	Reverse	Real-time PCR	GATGTAATGAATGAGTGCGTCTG
f13a1a	Forward	*In situ* hybridization	TGGAGGATTTCAACATTTACACAGCA
f13a1a	Reverse	*In situ* hybridization	ATGTTTCCACACAGACTGGAGTCT

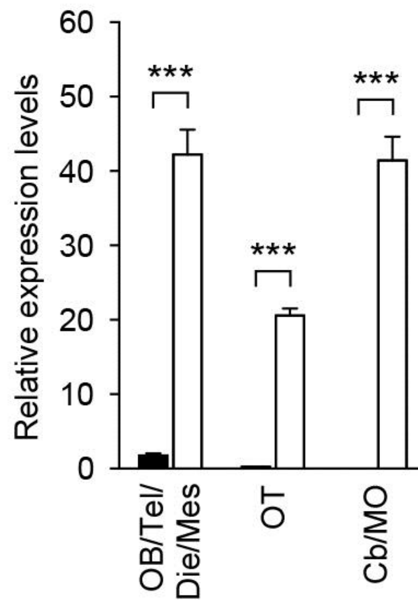

Figure 2-1. Sex differences in the expression level of *f13a1a* in three portions of the medaka brain: the olfactory bulb, telencephalon, diencephalon, and mesencephalon except the optic tectum (OB/Tel/Die/Mes); the optic tectum (OT); and the cerebellum and medulla oblongata (Cb/MO). The filled columns represent males and the open columns females. ***, $p < 0.001$ between the sexes in the same brain portion (unpaired t-test).

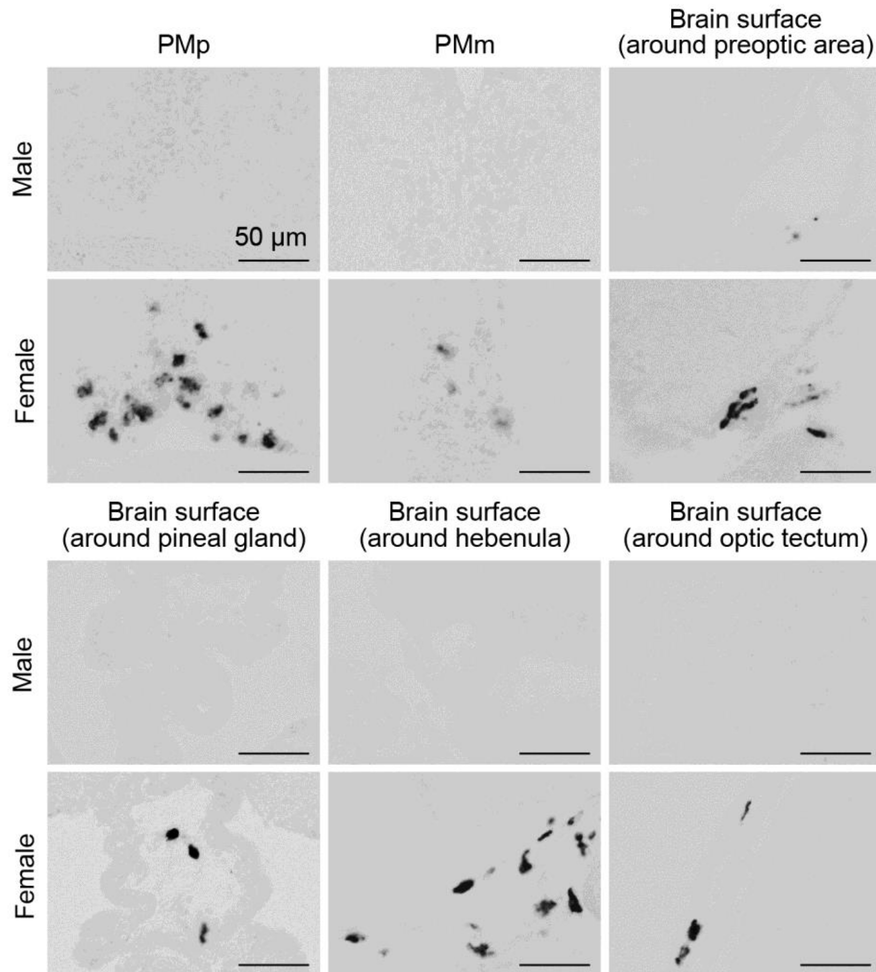

Figure 2-2. Distribution of *f13a1a* expression in the male and female medaka brain. Shown are representative micrographs of *f13a1a* expression in respective brain regions of both sexes. All scale bars represent 50 µm.

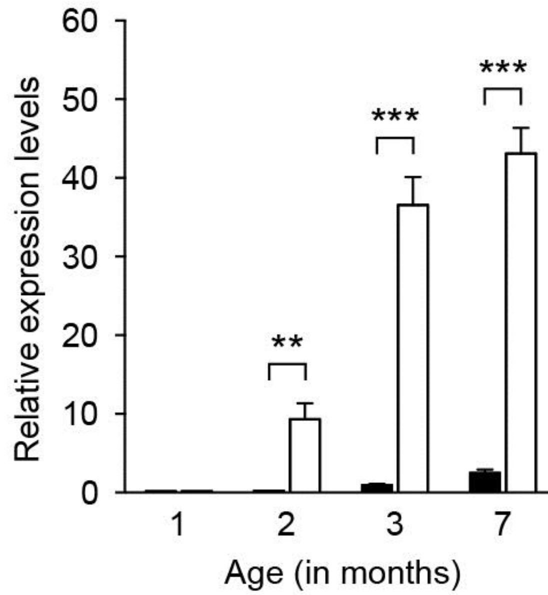

Figure 2-3. Sex differences in the overall expression of *f13a1a* in the medaka brain during growth and sexual maturation (from 1 to 7 months of age). The filled columns represent males and the open columns females. There were significant main effects of both sex and age and a significant interaction between these two factors ($p < 0.001$ for all). **, $p < 0.01$; ***, $p < 0.001$ between the sexes at the same age (Bonferroni's *post-hoc* test).

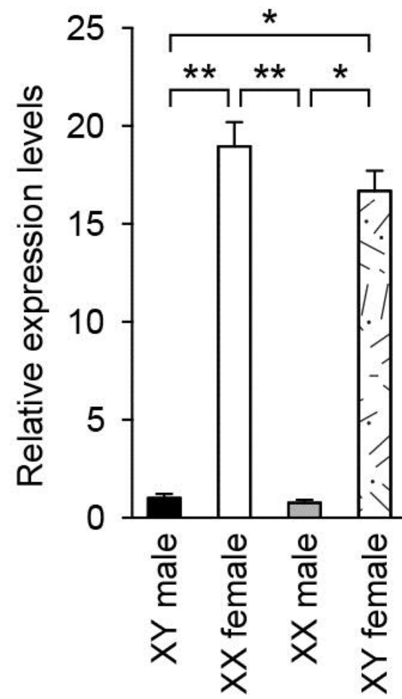

Figure 2-4. Genetic and phenotypic dependence of *f13a1a* expression in the medaka brain. The expression levels in sex-reversed XX gonadal males and XY gonadal females, as well as wild-type XY males and XX females, were examined and compared. *, $p < 0.05$; **, $p < 0.01$ (Dunn's *post-hoc* test).

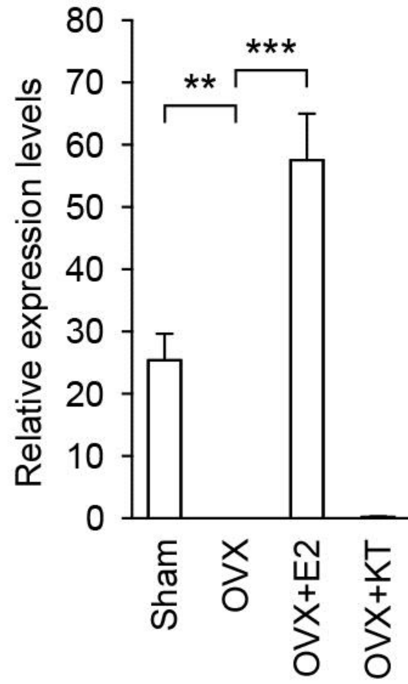

Figure 2-5. Effects of sex steroid hormones on *f13a1a* expression in the female medaka brain. The overall expression levels were measured in the brain of sham-operated females (Sham) and ovariectomized females that were exposed to the vehicle alone (OVX), E2 (OVX+E2), or 11KT (OVX+KT). **, $p < 0.01$; ***, $p < 0.001$ (Dunn's *post-hoc* test).

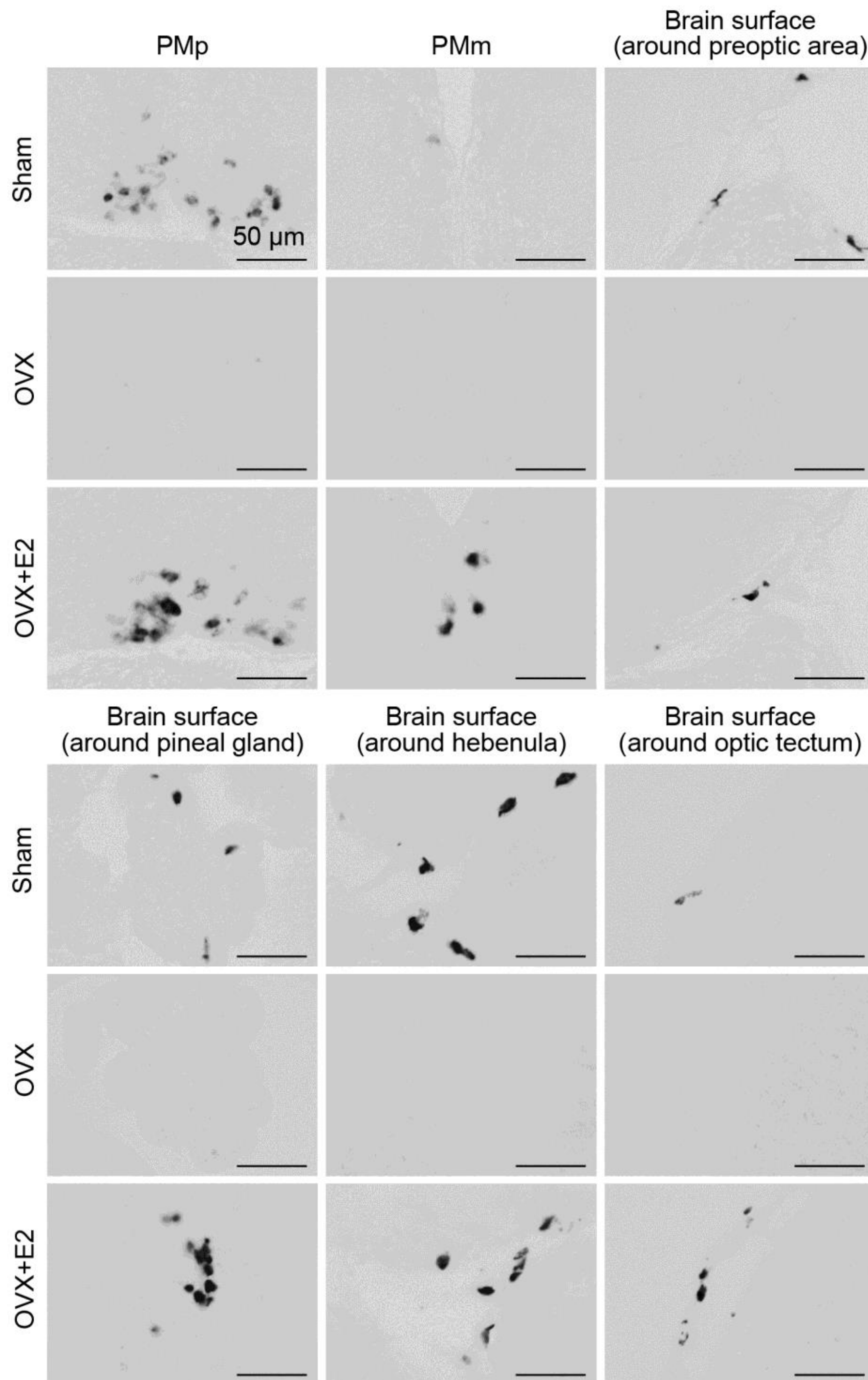

Figure 2-6. Effects of estrogen on *f13a1a* expression in respective brain regions of female medaka. Shown are representative micrographs of *f13a1a* expression in PMp, PMm, and the immediate vicinity of the brain surface of sham-operated females (Sham) and ovariectomized females that were exposed to the vehicle alone (OVX) or E2 (OVX+E2). All scale bars represent 50 μm.

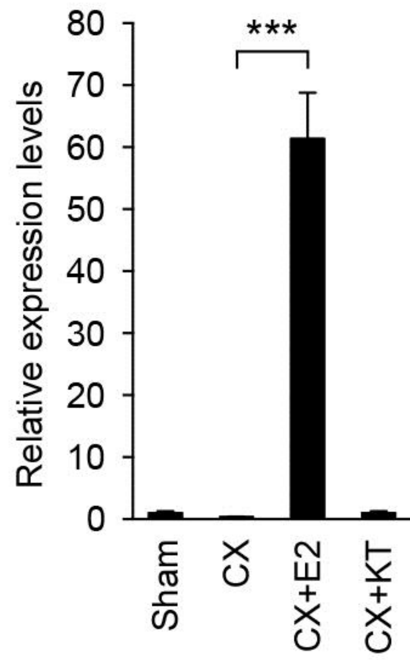

Figure 2-7. Effects of sex steroid hormones on *f13a1a* expression in the male medaka brain. The overall expression levels were measured in the brain of sham-operated males (Sham) and castrated males that were exposed to the vehicle alone (CX, E2 (CX+E2), or 11KT (CX+KT). ***, $p < 0.001$ (Dunn's *post-hoc* test).

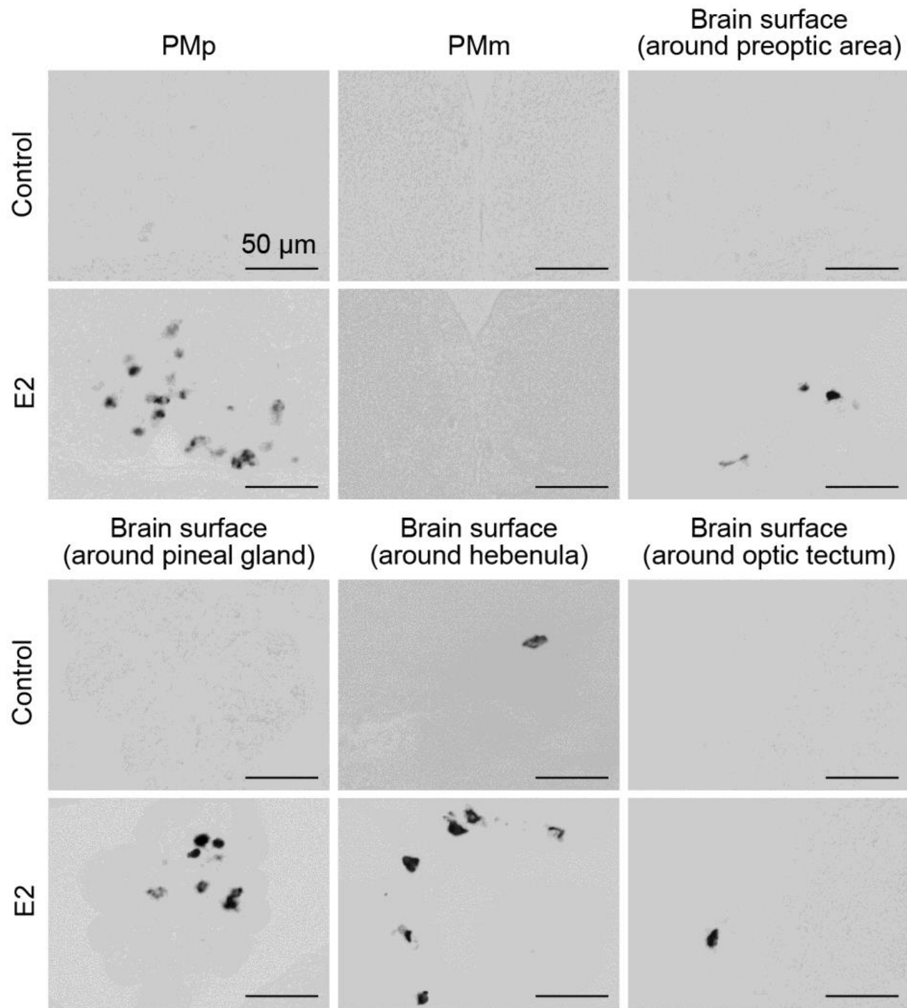

Figure 2-8. Effects of estrogen on *f13a1a* expression in respective brain regions of male medaka. Shown are representative micrographs of *f13a1a* expression in PMp, PMm, and the immediate vicinity of the brain surface of males with intact testis that were exposed to the vehicle alone (Control) or E2. All scale bars represent 50 µm.

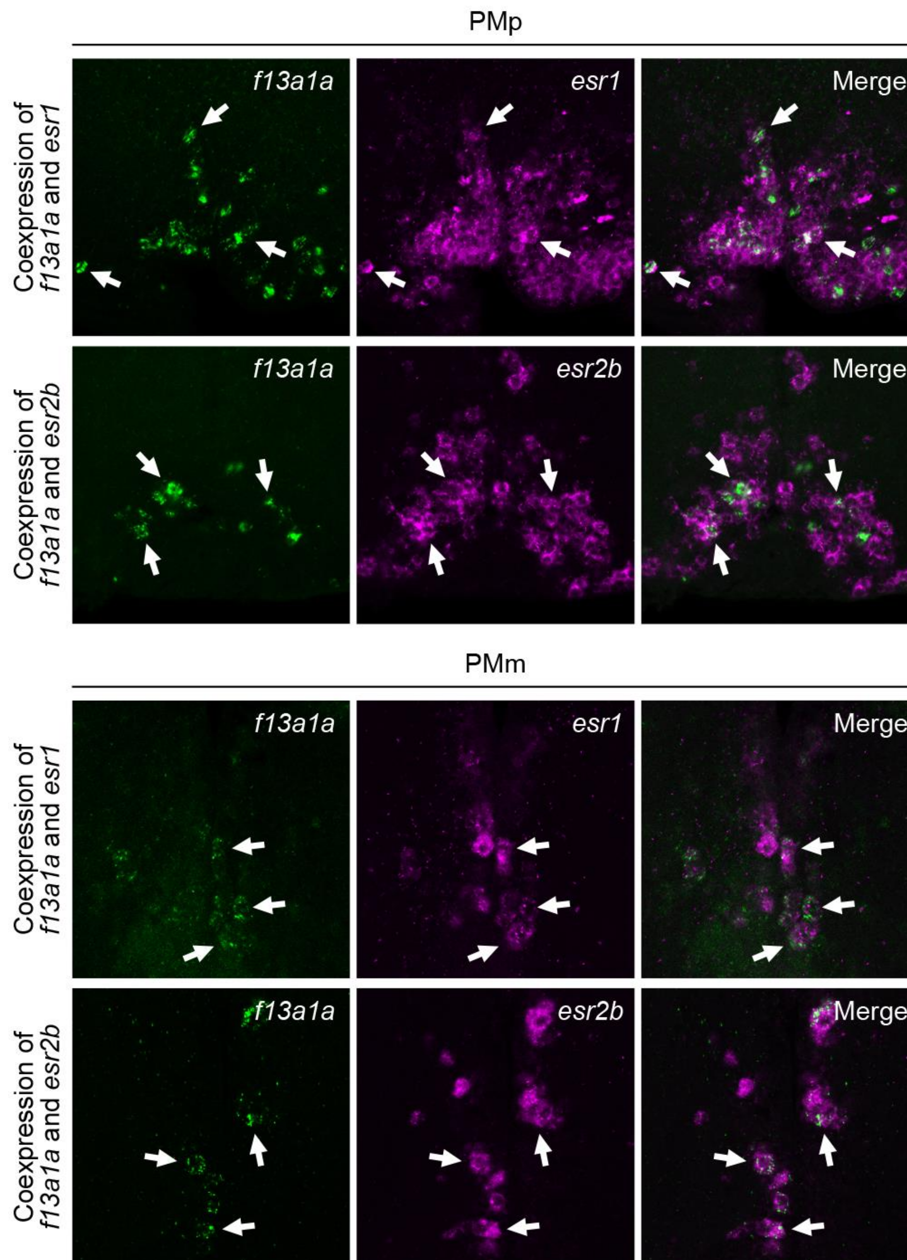

Figure 2-9. Expression of ER in female-specific *f13a1a*-expressing neurons. The possible coexpression of *f13a1a* and *esr1*/*esr2b* was examined in PMp and PMm of the female brain. Representative micrographs of PMp and PMm are depicted in the upper two and lower two rows, respectively. In each row, the left and middle panels show images of *f13a1a* (green) and *esr1*/*esr2b* (magenta) expression, respectively, in the same sections; the right panels show the merged images. Representative neurons coexpressing *f13a1a* and *esr1*/*esr2b* are indicated by arrowheads.

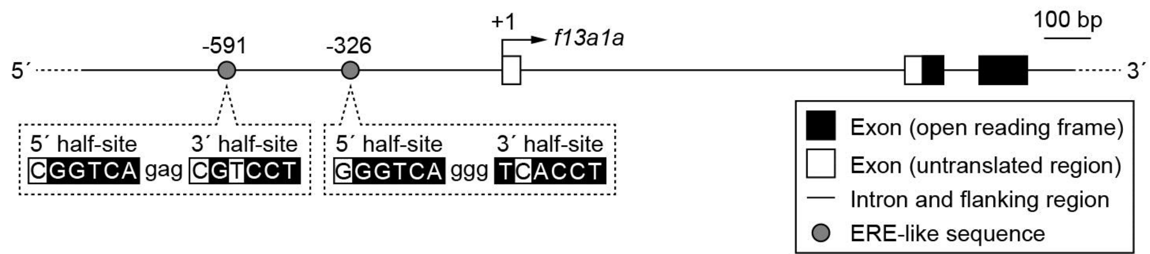

Figure 2-10. Schematic illustration of the location of two palindromic ERE-like sequences found in the 5′ proximal region of *f13a1a*. The bent arrow indicates the transcription start site. The nucleotide sequences of the likely EREs are shown; white letters on a black background indicate nucleotides identical to the consensus ERE sequence (AGGTCAnnnTGACCT).

Chapter 3

Unraveling the mechanisms underlying male-biased expression of *cntfa* and *pdlim3a* in the medaka brain

Introduction

In vertebrates, sex differences in the brain have been attributed to differences in gonadal hormone secretion; however, recent studies provide evidence that sex chromosome-linked genes, independent of gonadal hormones, also mediate sex differences in the brain (Arnold and Chen, 2009; McCarthy and Arnold, 2011; Arnold *et al*., 2013). In rodents, the sex-determining gene on the Y chromosome, *Sry*, has male-specific expression in the substantia nigra of the midbrain, where it directly influences motor performance by stimulating the expression of the dopamine-synthesizing enzyme tyrosine hydroxylase (Dewing *et al*., 2006). In zebra finches, the Z chromosome-specific gene *ntrk2* (also called *trkb*), which encodes a member of the neurotrophic tyrosine receptor kinase family, is expressed more abundantly in the male brain by virtue of its double genomic dose in males (Chen *et al*., 2005). Because Ntrk2 acts as a high-affinity receptor for brain-derived neurotrophic factor (BDNF), it is supposed to contribute to the masculinization of the neural song circuit.

In addition, several divergent gametologous gene pairs (homologous genes on opposite sex chromosomes) (García-Moreno and Mindell, 2000), including *Usp9x*/*Usp9y* and *Utx*/*Uty* in mice (Xu *et al*., 2002; 2005; 2008) and *chd1z*/*chd1w* and *pkciz*/*asw* in zebra finches (Agate *et al*., 2003; 2004), are expressed in the brain in a sex-specific manner, although the importance of their differential expression is not yet known. There is also accumulating evidence that, in rodents, some sex differences in neural and behavioral phenotypes, including aggressive and parental behaviors (Gatewood *et al*., 2006), response to noxious stimuli (Gioiosa *et al*., 2008), behavioral tendency related to addiction (Quinn *et al*., 2007), and social interactions (McPhie-Lalmansingh *et al*., 2008, Cox and Rissman, 2011), are influenced by the sex chromosome complement, as well as gonadal hormones, although the

genes and pathways responsible for sex differences in these phenotypes remain unknown.

Despite these findings in mammals and birds, there have been, to my knowledge, no reports of any sex chromosome-linked genes with sexually dimorphic expression in the brain of reptiles, amphibians, and fish. They differ from mammals and birds in that their sex chromosomes arose fairly recently and independently in each genus or even species (many of them even lack sex chromosomes entirely) (Kondo *et al.*, 2009; Marshall Graves and Peichel, 2010; Kikuchi and Hamaguchi, 2013). Because the sex chromosomes of reptiles, amphibians, and fish are still in the early stages of differentiation, their two sex chromosomes are morphologically indistinguishable and likely to be virtually identical, differing at one or a few loci. For instance, in a teleost fish, medaka (*Oryzias latipes*), the Y chromosome is genetically the same as the X chromosome except for the addition of a 258-kb sequence (Y-specific region) that includes the sex-determining gene *dmy* (Kondo *et al.*, 2006). This may explain why no evidence of sexually dimorphic expression of sex chromosome genes in the brain has been found in reptiles, amphibians, and fish.

However, in Chapter 1 of this thesis, I identified two gametologous genes with highly male-biased expression in the medaka brain: *cntfa*, which encodes ciliary neurotrophic factor a, and *pdlim3a*, which encodes PDZ and LIM domain 3 a. In this chapter, evidence was obtained that both of these genes are controlled by sex steroid hormones, suggesting that they may serve as focal points of the crosstalk between the genetic and hormonal pathways that direct the sexual differentiation of the brain.

Materials and Methods

Animals

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Tokyo. Medaka of the d-rR strain were maintained at 28°C with a 14-hour light/10-hour dark photoperiod and were fed 3 or 4 times per day with live brine shrimp and commercial pellet food (Otohime; Marubeni Nisshin Feed). Sexually mature adults of 3–5 months of age were sampled at 0.5–3 hours following the onset of light and used for analyses unless otherwise noted.

Spatial expression analysis of *cntfa* and *pdlim3a*

Sex differences in the spatial expression patterns of *cntfa* and *pdlim3a* in the brain were examined by real-time PCR. The whole brain was removed from male and female medaka (n = 6 for each sex) and divided into 3 portions: (i) the olfactory bulb, telencephalon, diencephalon, and mesencephalon except the optic tectum (OB/Tel/Die/Mes); (ii) the optic tectum (OT); and (iii) the cerebellum and medulla oblongata (Cb/MO). Total RNA was isolated separately from each portion using the RNeasy Lipid Tissue Mini Kit (Qiagen), and cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). PCR was performed using LightCycler 480 SYBR Green I Master on a LightCycler 480 System II (Roche). A melting curve analysis was conducted for every reaction to ensure that a single amplicon was produced in each sample. The expression of *actb* in each sample was used to normalize the expression of target transcripts by the standard curve method. Primers used for real-time PCR are listed in Table 3-1.

Temporal expression analysis of *cntfa* and *pdlim3a*

The expression profiles of *cntfa* and *pdlim3a* during growth and sexual maturation were also assessed by real-time PCR as described above using the whole brain of male and female medaka (n = 8 for each sex) at the following stages: 1 month of age, at which stage secondary sexual characteristics begin to appear; 2 months of age, at which stage fish are juvenile and have not yet spawned; 3 months of age, at which stage fish have become sexually mature and spawned; and 7 months of age, at which stage fish have regressed somewhat and the frequency of spawning has declined.

Gene dosage analysis

Genomic DNA was isolated separately from individual whole bodies of male and female medaka by proteinase K and RNase A treatment followed by extraction with phenol/chloroform/isoamyl alcohol and dialysis using a Float-A-Lyzer (Spectrum Laboratories, Rancho Dominguez, CA, USA). The resulting DNA was used as the template for real-time PCR to compare the gene dosage of *cntfa* and *pdlim3a* in the male and female

genomes. Primers used for real-time PCR are listed in Table 3-1.

Evaluation of genetic and phenotypic sex dependence of expression

I next investigated whether sex differences in *cntfa* and *pdlim3a* expression coincided with the genetic sex and/or phenotypic sex by producing and examining sex-reversed medaka. XX gonadal males and XY gonadal females were produced as described previously (Okubo *et al*., 2011). The whole brain was removed from these sex-reversed XX males and XY females and wild-type XY males and XX females, all of which were sexually mature and spawning (n = 8 for each group). Total RNA isolation, cDNA synthesis, and real-time PCR were performed as described above.

Evaluation of hormonal effects on expression

The testis was surgically removed from male medaka following the procedure described elsewhere (Kanda *et al*., 2008). Castrated fish were immersed in water containing 100 ng/ml of 11KT (n = 9) or E2 (n = 6) or the vehicle alone (n = 8) for 6 days as described previously (Nakasone *et al*., 2013). Sham-operated male fish (n = 8) treated with the vehicle alone were used as controls. The whole brain of these fish was dissected and used to address *cntfa* and *pdlim3a* expression by real-time PCR as described above.

Similarly, the ovary was removed from female medaka, which were then treated with 11KT, E2, or the vehicle alone as above for 5 days (n = 8 for each group). Sham-operated female fish (n = 8) were treated with the vehicle alone as controls. The expression of *cntfa* and *pdlim3a* in the brain of these fish was assessed by real-time PCR as described above.

Statistical analysis

All data are presented as the mean and the standard error of the mean. In real-time PCR analyses to quantify transcripts, the expression level of target transcripts (normalized to that of *actb*) in the brain of sexually mature adult males was arbitrarily set to 1, and the relative difference was calculated in order to facilitate comparisons among analyses.

Statistical analyses were performed using the GraphPad Prism software (GraphPad Software). Comparisons between two groups of data were evaluated for statistical

significance by the unpaired two-tailed Student's *t*-test. When the F-test indicated a significant difference in variance between groups, Welch's correction was applied. Comparisons between more than two groups were evaluated by one-way or two-way analysis of variance followed by Bonferroni's *post-hoc* test. In cases where the variances were significantly different among groups, data were log-transformed to normalize distributions prior to analysis.

Results

Sex differences in the spatial and temporal expression of *cntfa* and *pdlim3a* in the medaka brain

Levels of *cntfa* and *pdlim3a* expression in three portions of the brain were examined by real-time PCR and compared between the sexes (Figure 3-1). For both genes, comparable levels of expression were observed among the three brain portions. In addition, they showed comparable levels of male bias in expression among the brain portions.

The expression profiles of *cntfa* and *pdlim3a* during growth and sexual maturation were also analyzed in the whole brain (Figure 3-2). Their male-biased expression was evident as early as 1 month of age and persisted thereafter. Their expression levels gradually increased with growth and sexual maturation.

Gene dosage of *cntfa* and *pdlim3a* in the male and female medaka genomes

In order to define the mechanisms underlying the male-biased expression of *cntfa* and *pdlim3a*, I first examined and compared the gene dosage of these genes in the male and female genomes (Figure 3-3). No significant sex difference was observed in the *cntfa* dosage, whereas a slight (1.18-fold) but significant sex difference was detected in the *pdlim3a* dosage.

Genetic and phenotypic sex dependence of *cntfa* and *pdlim3a* expression

I then investigated whether sex differences in their expression coincided with genetic sex or phenotypic sex by producing sex-reversed fish and examining their expression in the brain of these fish as well as that of wild-type fish (Figure 3-4). Sex-reversed XY females and

wild-type XY males exhibited higher levels of *cntfa* and *pdlim3a* expression than sex-reversed XX males and wild-type XX females. Sex-reversed XY females showed slightly but significantly higher levels of *cntfa* expression and, in contrast, significantly lower levels of *pdlim3a* expression than wild-type XY males.

Effects of sex steroid hormones on *cntfa* and *pdlim3a* expression

In addition, the effects of sex steroid hormones on *cntfa* and *pdlim3a* expression in the brain were evaluated by means of a gonadectomy (castration for males and ovariectomy for females) followed by steroid hormone treatment. The expression of *cntfa* in males showed no significant response to any treatments (Figure 3-5). The expression of *pdlim3a* in males significantly increased by castration, and this effect was abolished by subsequent treatment with 11KT, whereas E2 had no such effects (Figure 3-5). In females, *cntfa* expression, although not affected by ovariectomy, increased with E2 treatment; 11KT had no such effects (Figure 3-6). However, *pdlim3a* expression did not show clear responses to ovariectomy or subsequent treatment with 11KT or E2 (Figure 3-6).

Discussion

In Chapter 1, I identified *cntfa* and *pdlim3a*, which were two gametologous genes with highly male-biased expression in the medaka brain. While previous studies identified several sex chromosome genes that were differentially expressed between the male and female brain in mammals and birds (Dewing *et al*., 2006; Chen *et al*., 2005; Xu *et al*., 2002; 2005; 2008; Agate *et al*., 2003; 2004), this study is the first to demonstrate sexually dimorphic expression of sex chromosome genes in the brain of ectothermic vertebrates. As opposed to the situation in mammals and birds, the sex chromosomes of ectothermic vertebrates, including medaka, are in the early stages of differentiation, and their two sex chromosomes generally appear essentially identical except at the sex-determining locus (Marshall Graves and Peichel, 2010). In light of this information, the location of *cntfa* and *pdlim3a* outside the sex-determining Y-specific region was somewhat unexpected.

This finding led me to investigate the mechanisms underlying the male-biased expression

of these X and Y chromosome-shared genes. One obvious possibility is that males possess additional copies of these genes in the Y-specific region, which contains a large gap in the genome assembly. However, this seems highly unlikely because genomic real-time PCR revealed no major sex differences in the dose of either *cntfa* or *pdlim3a* in the medaka genome, although a minimally but significantly higher value for *pdlim3a* was observed in males. The reason for this difference is not clear, but one possible explanation is that the Y allele may be PCR-amplified more efficiently than the X allele, for example, because of a difference in the nucleotide sequence between the alleles. In addition, both *cntfa* and *pdlim3a* were mapped to single loci outside the Y-specific region and not additionally to any unassembled scaffolds, further eliminating the possibility of the presence of additional copies on the Y chromosome.

I subsequently evaluated the genetic and phenotypic sex dependence of *cntfa* and *pdlim3a* expression, and we found that XY individuals of either phenotypic sex have more pronounced expression of these genes than XX individuals. The expression of both *cntfa* and *pdlim3a* thus appears to be strongly correlated with genetic sex, indicating that the Y allele confers higher expression than the X allele for both genes. This is in reasonable agreement with the apparent sex difference in their expression even before the onset of sexual maturation, which suggests that their expression is mainly dependent upon genetic rather than hormonal factors. It is generally accepted that the two sex chromosomes rapidly diverge mainly because of the continued accumulation of mutations on the non-recombining, heterogametic sex chromosome (Y or W) (Marshall Graves, 2006; Marshall Graves and Peichel, 2010). Therefore, some mutations leading to the enhancement of *cntfa* and *pdlim3a* expression may have occurred on the medaka Y chromosome, and further studies are needed to test this idea. Also generally accepted is that most mutations on the Y/W chromosome are deleterious, leading to reduced function or inactivation of the gene products and eventually to the degradation of the Y/W chromosome (Marshall Graves, 2006). In this context, my finding of the male-biased expression seems unique because the mutations on the Y chromosome would likely have activated the Y chromosome genes. An alternative explanation for the higher expression from the Y allele could be that both *cntfa* and *pdlim3a* are the targets of the sex-determining, Y-specific transcription factor *dmy*. However, this appears less likely

because the expression of *dmy* was hardly detected in the adult brain (Kataaki Okubo; personal communication).

I also found that XY females showed subtly but significantly higher levels of *cntfa* expression and, in contrast, lower levels of *pdlim3a* expression than XY males. This indicates that the expression of both *cntfa* and *pdlim3a* is dependent not only on genetic sex but also on phenotypic sex. My data further provide evidence for the significant influences of hormonal factors on *cntfa* and *pdlim3a* expression; *cntfa* is positively regulated by estrogen in females and *pdlim3a* is negatively regulated by androgen in males. The upregulation of *cntfa* by estrogen in females most likely accounts for its higher expression in XY females than in XY males. The downregulation of *pdlim3a* by androgen seems inconsistent with its higher expression in XY males than in XY females. This finding suggests that androgen may attenuate the magnitude of the sex difference in *pdlim3a* expression and that other sex-dependent hormonal factors, which remain to be identified, may also be involved in the regulation of *pdlim3a*. At the very least, these results demonstrate that certain sex chromosome genes are controlled by sex steroid hormones. Recently, a similar situation was reported in zebra finches, where the expression levels of a Z-linked gene, *ntrk2*, in their brain were affected by estrogen (Tang and Wade, 2012). Sex steroid-responsive sex chromosome genes such as *cntfa* and *pdlim3a* in medaka and *ntrk2* in zebra finches may play a role in integrating the genetic and hormonal pathways that direct the sexual differentiation of the brain.

The question then arises as to the functional significance of the male-biased expression of *cntfa* and *pdlim3a* in the brain. Given that sex-reversed XX males and XY females appear to be as fully fertile as normal XY males and XX females in medaka, sex differences in *cntfa* and *pdlim3a* expression are likely relevant to processes other than the control of gametogenesis. Their expression levels were found to be nearly equivalent among the examined brain portions, suggesting that both genes are expressed ubiquitously in the brain (I tried to evaluate their expression by *in situ* hybridization but failed to detect any signals, possibly because of their widespread expression at relatively low levels per cell). Accordingly, both *cntfa* and *pdlim3a* may play general rather than specific roles in the brain as discussed below, and they may act in a sex-dependent fashion.

CNTF/Cntf has been shown to play essential roles in a variety of processes in the nervous system, including the self-renewal and differentiation of neural stem cells during embryonic development and in the adult brain in normal and diseased/injured states (Sleeman *et al*., 2000; Bauer *et al*., 2007). It may be of interest to note here that the teleost brain displays widespread neurogenesis throughout adulthood (Zupanc and Sîrbulescu, 2011). This has often been attributed to an extremely high amount of aromatase, the rate-limiting enzyme in estrogen biosynthesis, in the adult teleost brain, of an order of magnitude 100–1,000-fold greater than that of mammals, because estrogen has the ability to stimulate neurogenesis (Diotel *et al*., 2010; Le Page *et al*., 2010). My data show that estrogen administration enhanced *cntfa* expression, whereas the removal of circulating estrogen by ovariectomy had no obvious effect. Therefore, it seems possible that *cntfa* functions downstream of estrogen that is locally produced by brain aromatase to stimulate neurogenesis. Considering that the gene encoding aromatase (*cyp19a1b*) and the genes indicative of cell proliferation and differentiation are expressed more highly in females than in males in the medaka brain (Okubo *et al*., 2011; Takeuchi and Okubo, 2013), the male-biased expression of *cntfa* may serve to reduce the female-biased acceleration of the cell life cycle in the medaka brain.

Although PDLIM3/Pdlim3 has been shown to be essential for the development of cardiac muscle (Pashmforoush *et al*., 2001), nothing is known about its role within the brain. Given that PDLIM3/Pdlim3 has been implicated in cytoskeletal assembly, it might be involved in the development and/or morphogenesis of brain cells. Future studies are needed to evaluate the functional significance of the sex difference in *pdlim3a* expression in the brain.

The sex chromosomes of ectothermic vertebrates, including teleost fish, have evolved simultaneously and independently in different genera or even species (Kondo *et al*., 2009; Marshall Graves and Peichel, 2010; Kikuchi and Hamaguchi, 2013). Consequently, each genus or species possesses distinct gametologous genes. It seems reasonable to assume that some of these genes exhibit sexually dimorphic expression in the brain in a genus- or species-specific fashion, as is the case for *cntfa* and *pdlim3a* in medaka. Ectothermic vertebrates display genus- or species-specific sex differences in diverse behavioral and physiological traits. Their gametologous genes with sex-dependent expression and/or function, besides the sex-determining genes, may possibly contribute to such differences.

In summary, this chapter strongly indicates that both of the two sex chromosome-linked genes in medaka, *cntfa* and *pdlim3a*, are more abundantly expressed from the Y allele than from the X allele and are controlled by sex steroid hormones. Evidence is accumulating that sex differences in the brain are established by the effects of both sex-dependent hormones and sex chromosome complements. Therefore, gametologous genes with sex-dependent expression patterns, such as *cntfa* and *pdlim3a*, may serve as focal points of the crosstalk between the genetic and hormonal pathways underlying sex differences in the brain.

Table 3-1. Primers used in Chapter 3.

Target	Direction	Purpose	Sequence (5′ to 3′)
cntfa	Forward	Real-time PCR (brain)	AGAAGTGCTCGACGATGAGAAG
cntfa	Reverse	Real-time PCR (brain)	GGCATGGAGCGCAGTAATTT
cntfa	Forward	Real-time PCR (genome)	TGCTTCATAGGTTGATTGATTGTC
cntfa	Reverse	Real-time PCR (genome)	CTGTTTGGTGAAGGCAGGGTA
pdlim3a	Forward	Real-time PCR (brain)	AACATCCAAGATGCCATGGAG
pdlim3a	Reverse	Real-time PCR (brain)	CACGTCAGATTCCTCGATGCT
pdlim3a	Forward	Real-time PCR (genome)	GCTGCAGAAAGACCAGGATATG
pdlim3a	Reverse	Real-time PCR (genome)	CGTCGCTGTCGATAAACTCCT
actb	Forward	Real-time PCR (brain)	GCCTCAAAGCTATTATTTTACTCAACT
actb	Reverse	Real-time PCR (brain)	GATGTAATGAATGAGTGCGTCTG

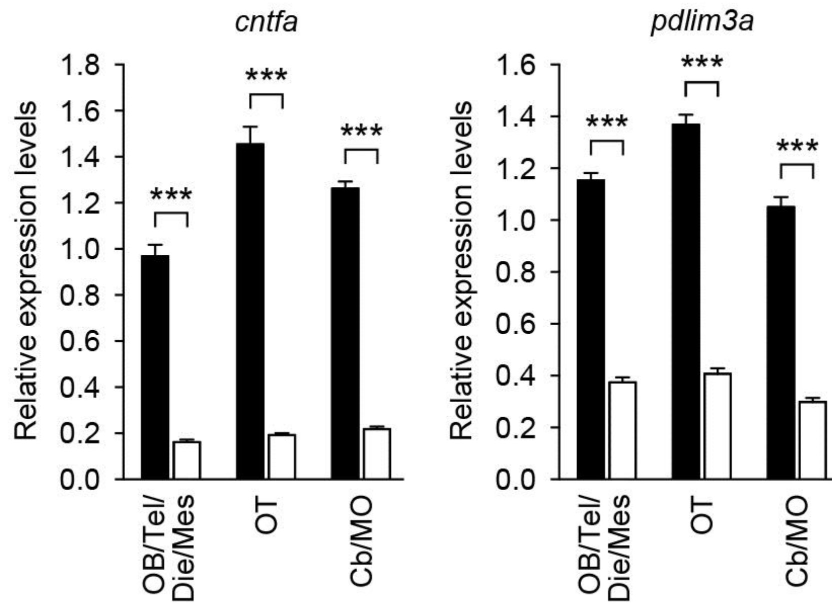

Figure 3-1. Sex differences in the levels of *cntfa* and *pdlim3a* expression in three portions of the medaka brain: the olfactory bulb, telencephalon, diencephalon, and mesencephalon except the optic tectum (OB/Tel/Die/Mes); the optic tectum (OT); and the cerebellum and medulla oblongata (Cb/MO). The filled columns represent males and the open columns females. ***, $p < 0.001$ between the sexes in the same brain portion (unpaired *t*-test).

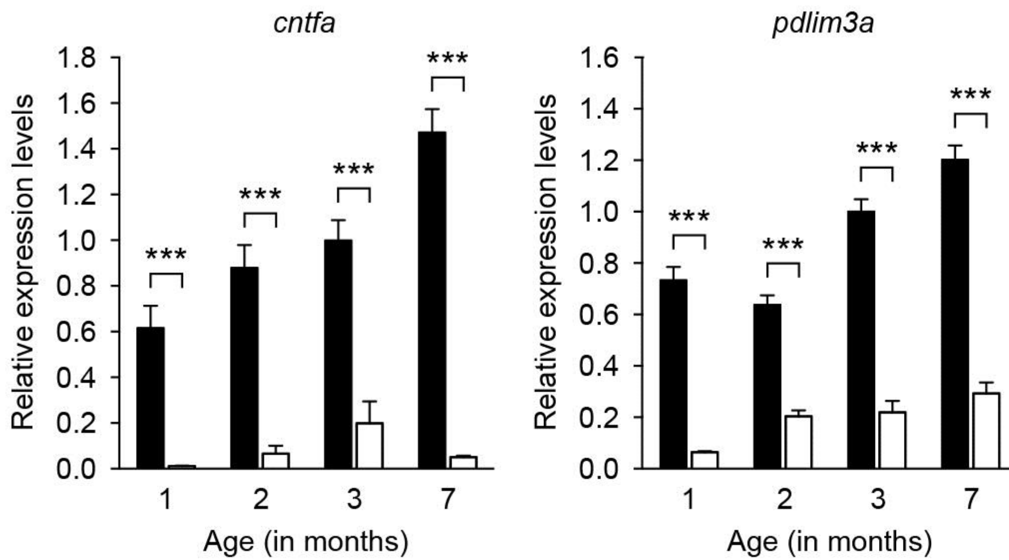

Figure 3-2. Sex differences in the levels of *cntfa* and *pdlim3a* expression in the medaka brain during growth and sexual maturation (from 1 to 7 months of age). The filled columns represent males and the open columns females. There were significant main effects of both sex and age and a significant interaction between these two factors ($p < 0.001$ for all). ***, $p < 0.001$ between the sexes at the same age (Bonferroni's *post-hoc* test).

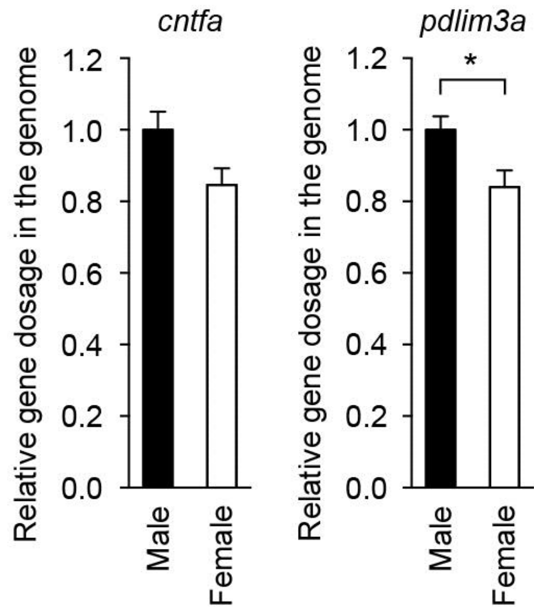

Figure 3-3. Comparison of the gene dosage of *cntfa* and *pdlim3a* between the male and female medaka genomes. *, $p < 0.05$ (unpaired *t*-test).

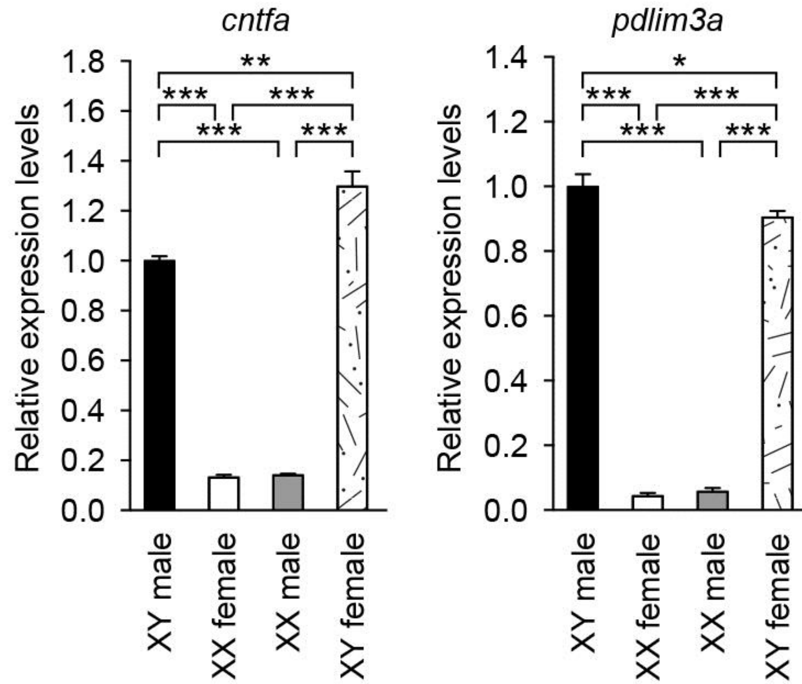

Figure 3-4. Genetic and phenotypic dependence of *cntfa* and *pdlim3a* expression in the medaka brain. The expression levels in sex-reversed XX gonadal males and XY gonadal females, as well as wild-type XY males and XX females, were examined and compared. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Bonferroni's *post-hoc* test).

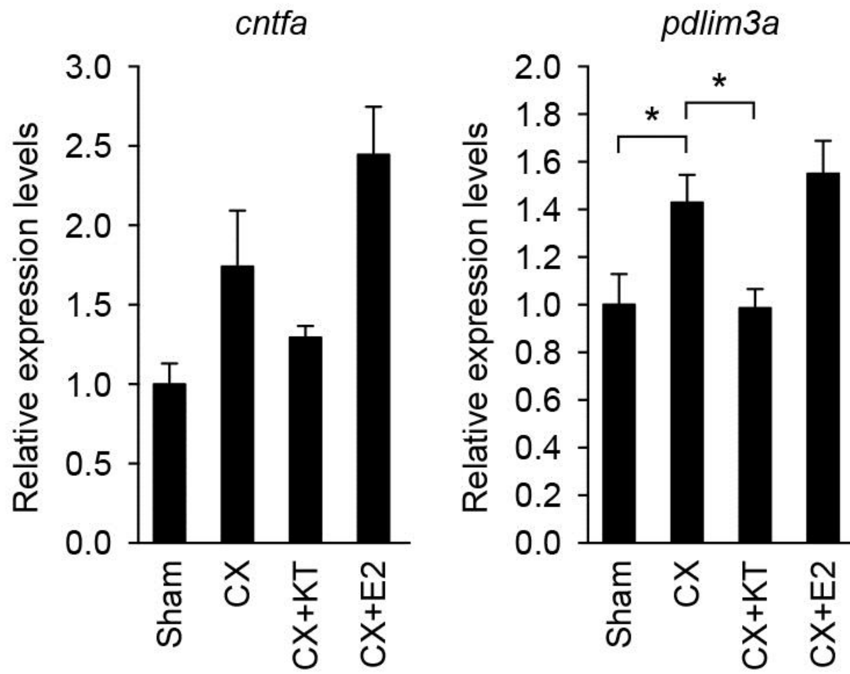

Figure 3-5. Effects of sex steroid hormones on *cntfa* and *pdlim3a* expression in the male medaka brain. The expression levels of *cntfa* and *pdlim3a* were measured in the brain of sham-operated males (Sham) and castrated males that were exposed to the vehicle alone (CX), 11-ketotestosterone (11KT) (CX+KT), or E2 (CX+E2). *, $p < 0.05$ (Bonferroni's *post-hoc* test).

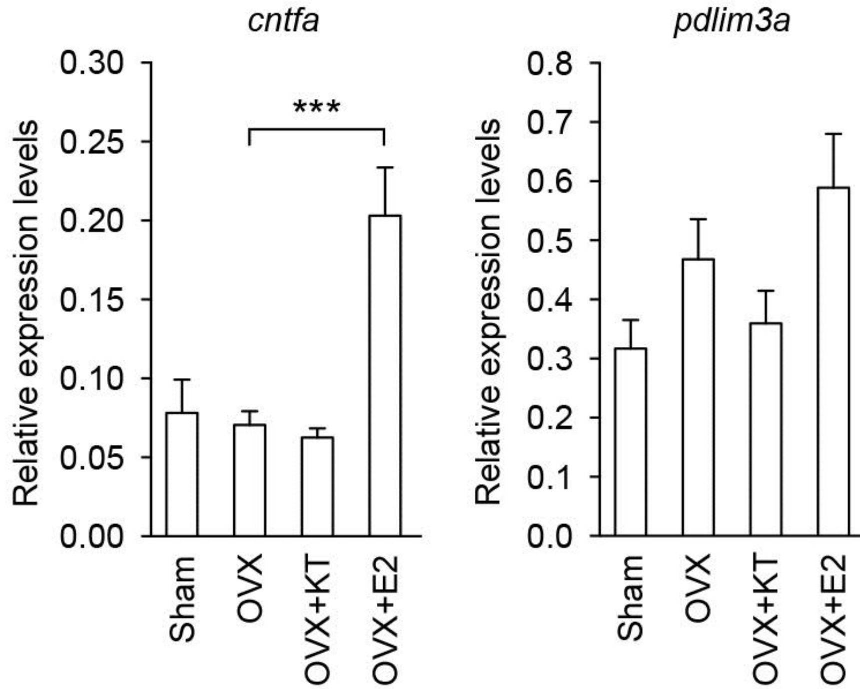

Figure 3-6. Effects of sex steroid hormones on *cntfa* and *pdlim3a* expression in the female medaka brain. The expression levels of *cntfa* and *pdlim3a* were measured in the brain of sham-operated females (Sham) and ovariectomized females that were exposed to the vehicle alone (OVX), 11KT (OVX+KT), or E2 (OVX+E2). ***, $p < 0.001$ (Bonferroni's *post-hoc* test).

General Discussion

Studies in rodents during the past half-century have repeatedly shown that the brain undergoes an irreversible process of sexual differentiation during perinatal development (Phoenix *et al*., 1959; McDonald *et al*., 1970; MacLusky and Naftolin, 1981). More specifically, androgen secreted from the fetal testis reaches the brain of genetic males, and is then converted to estrogen by the enzyme aromatase, which subsequently initiates the irreversible process of brain masculinization. This process has been referred to as the general concept of sexual differentiation of the brain. However, a reappraisal of the concept developed by rodent studies is now clearly necessary in other vertebrate species, as several studies on non-rodent species have challenged this concept and provided evidence for alternative mechanisms. For example, it has been shown that in mammals and birds, sex chromosome-linked genes also mediate sex differences in the brain, independent of gonadal hormones, as described in General Introduction (Agate *et al*., 2003; Arnold, 2009a; 2009b; Arnold and Chen, 2009; McCarthy and Arnold, 2011; Maekawa *et al*., 2013). Moreover, the brain of teleost fish (even species that never undergo spontaneous sex changes under natural conditions) exhibits conspicuous sexual plasticity throughout lifetime (Howell *et al*., 1980; Stacey and Kyle, 1983; Stacey and Kobayashi, 1996; Paul-Prasanth *et al*., 2013; Takatsu *et al*., 2013). Given this situation, teleost fish (medaka) was chosen as the subject of this study, with the expectation that some unique features in teleosts, including thoroughgoing sexual plasticity of the brain and sex chromosomes that evolved independently of those in mammals and birds, should allow me to obtain unique information that was otherwise unavailable.

I first searched for genes differentially expressed between males and females in the medaka brain. Microarray analysis led to the identification of three previously unreported sexually dimorphic genes: a female-predominant gene *f13a1a* (encoding a member of the transglutaminase family, F13a1a) and two male-predominant genes *cntfa* (encoding a polypeptide hormone belonging to the interleukin-6 family of cytokines, Cntfa) and *pdlim3a* (encoding a member of the PDZ-LIM protein family, Pdlim3a).

f13a1a exhibits the larger sex difference in overall expression in the brain than any other autosomal genes examined in any vertebrate species. Evidence was obtained that *f13a1a* is

female-specifically expressed in neurons in two preoptic nuclei, PMp and PMm, which have been implicated in the control of pituitary hormone secretion and reproductive behavior. These findings lead me to assume that *f13a1a* may contribute to sex differences in these processes, although this idea remains speculative as no function has been defined for F13A1/F13a1 in neurons. I am currently planning to generate *f13a1a*-deficient medaka by transcription activator-like effector nucleases (TALENs) (Joung and Sander, 2013) and examine their phenotype. In Addition, I am planning to examine the expression of several neuropeptides which have been implicated in pituitary hormone secretion and repoductive behavior and are presumably coexpressed with *f13a1a* in the same neurons, in order to address the relationship between *f13a1a* and these neuropeptide. These analyses will shed light on the physiological role of *f13a1a* and the significance of its female-specific expression. It may also be worth examining sex differences in the expression of the *f13a1a* paralog, *f13a1b*, for which no information is currently available.

A key finding in this study is that the sexually dimorphic expression of *f13a1a* results from stimulatory effects of estrogen secreted by the ovary and is completely reversible even in adulthood. Recent work from our research group has identified several other genes that are expressed in the medaka brain in a female-biased fashion, including *cyp19a1b*, *esr2b*, *arb* (encoding an androgen receptor (AR) subtype, Arb), *hebp3*, and *npb* (Okubo *et al*., 2011, Hiraki *et al*., 2012; 2014; Nakasone *et al*., 2013). The female-biased expression of all these genes is caused by ovarian estrogen in a reversible manner, as is the case for *f13a1a*. This is in striking contrast to the situation in the rodent brain, where sex differences in the expression of several genes, including galanin, cholecystokinin, substance P, vasopressin, ER, and progestin receptor, are epigenetically programmed in an irreversible manner by the action of sex steroids during perinatal development (Polston and Simerly, 2003; Westberry *et al*., 2010; Schwarz *et al*., 2010; Auger *et al*., 2011). This irreversible process most likely causes permanent changes in the expression of these genes and consequently brain function as well in a sex-dependent manner, and so does the rodent brain. It seems reasonable to assume that the absence of this process in the teleost brain account for its marked sexual plasticity throughout lifetime. In other words, the lifelong sexual plasticity of the teleost brain is most likely attributable, at least in part, to the reversibility of sexual dimorphism in gene expression

in the brain, depending on the sex steroid milieu, even in adulthood. Importantly, these genes include the receptors of sex steroids, *esr2b* and *arb* (Hiraki *et al*., 2012), indicating that even in adulthood, sexually differentiated sensitivity of the teleost brain to sex steroids can be reversed between the sexes by sex steroids themselves, which should in turn lead to the reversal of sex differences in the expression of sex steroid-responsive genes. Such a mechanism presumably forms the basis of the persistent sexual plasticity of the teleost brain.

My data also suggest that estrogen, bound to Esr1 and/or Esr2b, directly activate the transcription of *f13a1a* through the canonical ER-ERE pathway in the PMp and PMm neurons. To further strengthen this supposition, I am now planning to validate the response of the *f13a1a* promoter to estrogen and the functionality of the ERE-like sequences identified in this study. Despite the widely held view that estrogen affects the expression of many neural genes, only several have been shown to be under the control of the ER-ERE pathway (Hudgens *et al*., 2009). The results obtained from these studies will thus be important in understanding the impact of estrogen on neural gene expression. Also found in this study is that the direct transcriptional activation of *f13a1a* by estrogen is likely conserved among teleost species, suggesting a conserved role for this gene in brain sexual differentiation in teleosts. Given that the phenotype of *F13a1*-deficient mice is in part sex-specific, with only males exhibiting cardiac impairment in adulthood (Souri *et al*., 2008), this conservation may extend across vertebrates. Further studies, especially comparative analyses in different species, would help test this idea.

Another important finding in the present study related to *f13a1a* was that this gene is expressed in cells lying immediately outside the brain parenchyma and these cells are present much more abundantly in females than males. Although it remains to be determined what type of cells they are and what extent this phenomenon is conserved among vertebrates, this finding argues that sex differences exist not only in the brain parenchyma but also in its accompanying cells. In line with this, a recent study in our research group has shown that *hebp3* is expressed in a female-biased manner in the meninges, the membrane enveloping the brain parenchyma, of medaka (Nakasone *et al*., 2013). It therefore seems that, in brain sex-related studies, future attention should be paid to cells surrounding the brain as well as the brain parenchyma.

The other two sexually dimorphic genes focused on in this thesis, *cntfa* and *pdlim3a*, are both expressed in a male-biased manner and located on sex chromosomes. These genes are present on both X and Y chromosomes (in a region that is shared by the X and Y chromosomes). Based on my results, the most likely mechanism for their male-biased expression is that the Y allele confers higher expression than the X allele for both genes. There may be differences in the nucleotide sequences of these two alleles, perhaps in the core promoter region or enhancer region that interact with transcription factors, and these differences may be responsible for the male-biased expression of these genes. Both X and Y alleles of these genes should be fully sequenced and compared to identify such differences.

The contribution of sex chromosome complement to sexual differentiation of the brain independently of gonadal hormones has been a hot topic during the past decade (Davies and Wilkinson, 2006; Abel and Rissman, 2012; Arnold *et al*., 2013). Several sex chromosome-linked genes that are expressed in the brain in a sex-biased manner, including *Sry*, *ntrk2*, *Usp9x*/*Usp9y*, *Utx*/*Uty*, *chd1z*/*chd1w*, and *pkciz*/*asw*, have been identified in rodents and zebra finches (Xu *et al*., 2002; 2005; 2008; Agate *et al*., 2003; 2004; Chen *et al*., 2005; Dewing *et al*., 2006), although the importance of their differential expression is still unknown, except for *Sry* in rodents, which male-specifically facilitate motor performance (Dewing *et al*., 2006). However, there have been no reports of any sex chromosome genes with sex-biased expression in the brain of reptiles, amphibians, and fish, probably because their sex chromosomes are still in the early stages of differentiation and are virtually identical, differing at one or a few loci (Marshall Graves and Peichel, 2010). In this study, I provide evidence that sexually dimorphic expression of sex chromosome genes in the brain is a conserved phenomenon across vertebrates.

Now the important question is to what extent and how sex chromosome genes contribute to sexual differentiation of the vertebrate brain. As described above, *Sry* is the only sex chromosome gene whose function in the brain has been uncovered (Dewing *et al*., 2006) and, therefore, this question remains largely unanswered. The functional significance of the male-biased expression of *cntfa* and *pdlim3* in the medaka brain also remains to be determined. If these genes have significant functions in the medaka brain, these could be disclosed by analyzing the phenotypes of XX and XY medaka of the same gonadal type (XX

males versus XY males and XX females versus XY females). Considering that sex-reversed XX gonadal males and XY gonadal females appear to be as fully fertile as normal males and females, respectively, in medaka, sex differences in *cntfa* and *pdlim3a* expression are likely relevant to processes other than those related to sex chromosomes as gametogenesis. I supposed that no attempt has been made to investigate in detail possible differences between the phenotypes of XX and XY medaka of the same gonadal type. It is necessary to examine and compare their phenotypes from various points of view in the near future.

The sex chromosomes of mammals and birds have evolved independently sometime after their divergence and therefore are not homologous. Moreover, the sex chromosomes of other vertebrates have evolved independently in each genus or even species (Kondo *et al*., 2009; Marshall Graves and Peichel, 2010; Kikuchi and Hamaguchi, 2013), which has led to the current situation that each genus or species possesses distinct sex chromosomes and, as a consequence, distinct sex chromosome-linked genes. Vertebrates more or less display genus- or species-specific sex differences in behavioral and physiological traits. Taken together, I have proposed an idea that these differences may be attributable, at least in part, to genus- or species-specific sex chromosome genes with sex-dependent expression in the brain. In addition, the genera and species with evolutionarily older sex chromosomes would display larger sex differences, because more extensive divergence should have accumulated between the two alleles of sex chromosome genes in their genomes.

At the same time, however, the situation appears not to be that simple, as I show here that the expression of *cntfa* and *pdlim3a* is genetically regulated in a male-biased manner, due to the higher expression from the Y allele, but is hormonally regulated in a female-biased manner, *i.e*., stimulated by estrogen and suppressed by androgen. This finding indicates that sex differences derived from sex chromosome complement may serve to reduce the differences caused by sex steroid hormones. de Vries (2004) pointed out that some sex differences in the brain should serve to prevent, but not to cause, sex differences in brain functions and behavior, by compensating for existing sex differences in physiological conditions (*e.g*., gonadal hormone levels that may generate undesirable sex differences). Our data strongly suggest that this actually occurs in sex chromosome genes.

Through the work in this thesis, I aimed to understand the development, maintenance,

and plasticity of sex differences in the teleost brain at the gene expression level. My data provided definitive evidence that sexually dimorphic gene expression in the teleost brain is highly plastic even in adulthood and that this plasticity relies on the sex steroid milieu. In rodents, sex steroid secreted by the gonad early in life causes irreversible and permanent sex differences in the brain. In contrast, the teleost brain exhibits a considerable degree of sexual plasticity throughout lifetime. Accordingly, it may be possible to assume that the teleost brain remains in the state corresponding to the early developmental stage in rodents, even in adulthood. My data also suggested that some genus- or species-specific sex differences in behavioral and physiological traits may be attributable to sex chromosome genes with sex-dependent expression in the brain. Further studies evaluating these concepts would be of considerable value in identifying what is general across vertebrates and what is species- or class-specific in the process of brain sexual differentiation.

Finally, it should be noted that a number of genes identified by microarray screening remain uncharacterized. Some genes seem to be worth further investigation; these include, for example, the genes encoding hypothalamic neuropeptide implicated in food intake, membrane-bound steroid receptor, transcription factor involved in neuronal differentiation, steroidogenic enzyme, heat-shock protein, serotonin receptor, and hormone transporter. Future studies should necessarily evaluate whether these genes indeed show sexually dimorphic expression in the brain and, if so, investigate their detailed expression pattern, regulatory mechanisms, and physiological function. These studies will help further understand sexual differentiation of the brain and physiological/behavioral traits in vertebrates, providing further insights into what is general across vertebrates and what is species- or class-specific in the process of sexual differentiation of the brain.

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