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Sexually dimorphic expression of the sex chromosome-linked genes *cntfa* and *pdlim3a* in the medaka brain

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ABSTRACT

In vertebrates, sex differences in the brain have been attributed to differences in gonadal hormone secretion; however, recent evidence in mammals and birds shows that sex chromosome-linked genes, independent of gonadal hormones, also mediate sex differences in the brain. In this study, we searched for genes that were differentially expressed between the sexes in the brain of a teleost fish, medaka (*Oryzias latipes*), and identified two sex chromosome genes with male-biased expression, *cntfa* (encoding ciliary neurotrophic factor a) and *pdlim3a* (encoding PDZ and LIM domain 3 a). These genes were found to be located 3–4 Mb from and on opposite sides of the Y chromosome-specific region containing the sex-determining gene (the medaka X and Y chromosomes are genetically identical, differing only in this region). The male-biased expression of both genes was evident prior to 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 lower vertebrates, including teleost fish, have evolved independently in different genera or species, sex chromosome genes with sexually dimorphic expression in the brain may contribute to genus- or species-specific sex differences in a variety of traits.

1. 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 [1,2]. 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 [3]. In zebra finches, the Z chromosome-specific gene *ntrk2* (or *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 [4]. 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) [5], including *Usp9x*/*Usp9y* and *Utx*/*Uty* in mice [6–8] and *chd1z*/*chd1w* and *pkciz*/*asw* in zebra finches [9,10], 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 [11], response to noxious stimuli [12], behavioral tendency related to addiction [13], and social interactions [14,15], 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.

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Despite these findings in mammals and birds, there have been, to our knowledge, no reports of any sex chromosome-linked genes with sexually dimorphic expression in the brain of lower vertebrates, including reptiles, amphibians, and fish. Lower vertebrates 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) [16–18]. 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* [19]. This may explain why no evidence of sexually dimorphic expression of sex chromosome genes in the brain has been found in lower vertebrates.

However, in this study, we 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. Evidence was also 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.

2. Materials and methods

Full description of the Materials and methods are available in the Supplementary information.

2.1. Animals

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Tokyo. Sexually mature adult medaka of 3–5 months of age were sampled at 0.5–3 h following the onset of light and used for analyses unless otherwise noted.

2.2. Microarray-based screening and molecular characterization

A high-density oligonucleotide microarray containing 385,000 probes (60-mer probes) representing 63,698 non-redundant medaka transcripts was designed and manufactured by Roche Diagnostics Japan (Tokyo, Japan). Microarray analysis was performed using total RNA isolated from the whole brain along with the pituitary of male and female medaka by Roche Diagnostics Japan. Differential expression of several transcripts that were identified by microarray profiling was examined using real-time PCR.

We focused on two transcript sequences (*cntfa* and *pdlim3a*) that were identified in the screen as having high male biases. The full-length sequences of these transcripts were determined by *in silico* cloning and rapid amplification of cDNA ends. The resulting sequences were subjected to phylogenetic tree construction using the neighbor-joining algorithm, and mapped on medaka chromosomes using the Ensembl BLAST server (http://www.ensembl.org/Multi/blastview).

2.3. Spatial and temporal expression analysis

The whole brain was removed from male and female medaka 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). Sex differences in the expression of *cntfa* and *pdlim3a* in these brain portions were examined by real-time PCR. The expression profiles of *cntfa* and *pdlim3a* during growth and sexual maturation were also assessed by real-time PCR using the whole brain of male and female medaka at 1, 2, 3, and 7 months of age.

2.4. Gene dosage analysis

Genomic DNA isolated separately from individual whole bodies of male and female medaka was used as the template for real-time PCR to compare the gene dosage of *cntfa* and *pdlim3a* in the male and female genomes.

2.5. Evaluation of genetic and hormonal sex dependence of expression

The whole brain was removed from sex-reversed XX males and XY females as well as wild-type XY males and XX females and subjected to real-time PCR to examine the expression of *cntfa* and *pdlim3a*.

Gonadectomized male and female medaka were treated with 100 ng/ml of 11-ketotestosterone (11KT; the most prominent, non-aromatizable teleost androgen) or estradiol-17β (E2) or the vehicle alone. Males were treated for 6 days while females were treated for 5 days. Sham-operated fish treated with the vehicle alone were used as controls. The whole brain of these fish was dissected and used to address the expression of *cntfa* and *pdlim3a* by real-time PCR.

3. Results

3.1. Identification of cntfa and pdlim3a as gametologous genes exhibiting male-biased expression 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 (Fig. 1A). These differentially expressed transcripts included *npb*, which has been identified as being expressed in a female-biased manner in the medaka brain by a subtractive screen [20], verifying the validity of the analysis. In this study, we focused on two 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 M50209 and M25981 showed 5.6- and 3.6-fold higher expression, respectively, in males (Fig. 1B and C).

Determination of the full-length sequences and the subsequent BLAST search revealed that the proteins encoded by M50209 and M25981 were most similar to known CNTF/Cntf and PDLIM3/Pdlim3, respectively (Fig. 1D and E). Phylogenetic analysis demonstrated that M50209 and M25981 encoded a medaka ortholog of CNTF/Cntf and PDLIM3/Pdlim3, respectively, in other vertebrate species (Fig. 1F and G). This analysis also revealed that two paralogs were 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 study were designated Cntfa and Pdlim3a). The sequence of the medaka *cntfa* was deposited in GenBank under Accession No. AB894420.

Both *cntfa* and *pdlim3a* were mapped to the sex chromosome (chromosome 1). 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 (Fig. 1H).

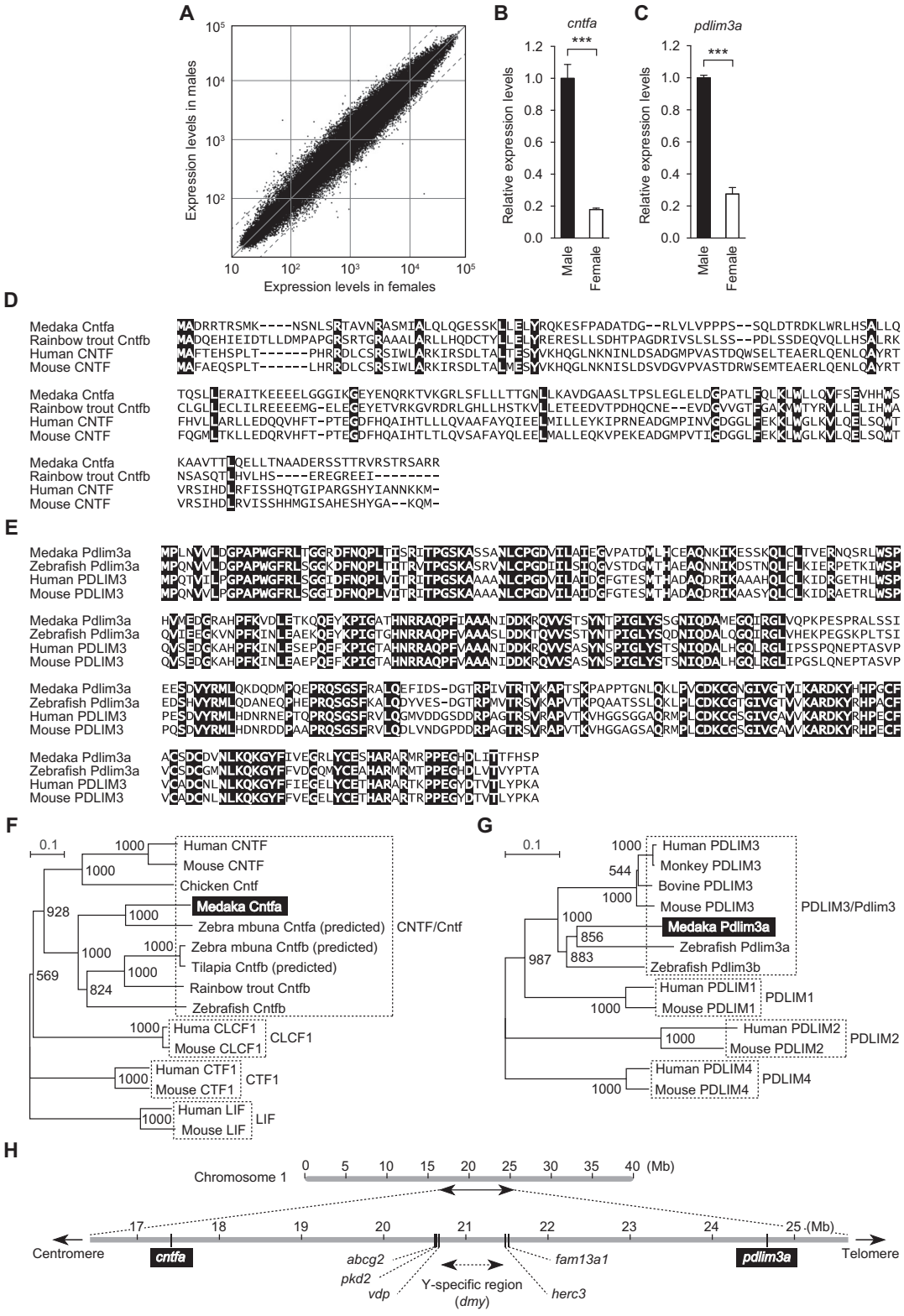

Fig. 1. Identification and molecular characterization of the medaka *cntfa* and *pdlim3a*. (A) Scatter plot comparing the expression profiles that were obtained by microarray analysis with the male (*Y*-axis, log scale) and female (*X*-axis, log scale) brain. Each dot represents one transcript (a set of probes) on the array. The solid diagonal line indicates no difference between the male and female brain, and the dashed lines indicate the threefold 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. (B and C) Verification of the male-biased expression of two transcripts, M50209 (*cntfa*) (B) and M25981 (*pdlim3a*) (C), in the brain by real-time PCR. $^{***}p < 0.001$ (unpaired *t*-test). (D and E) Alignments of deduced amino acid sequences of Cntf (D) and Pdlim3 (E) in medaka and other species. Identical amino acids are shown in white letters on a black background. (F and G) Phylogenetic analyses of CNTF/Cntf (F) and PDLIM3/Pdlim3 (G). 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 both Cntf and Pdlim3 in the teleost lineage, they were designated Cntfa/Cntfb and Pdlim3a/Pdlim3b (the products of the medaka transcripts that were identified in this study were designated Cntfa and Pdlim3a). (H) Location of *cntfa* and *pdlim3a* on the medaka sex chromosome (chromosome 1; 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* [19]. The Y-specific region has not been precisely mapped, but it is known to lie between *abcg2*/*pkd2*/*vdp* and *herc3*/*fam13a1*.

3.2. 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 (Fig. 2A and B). 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 (Fig. 2C and D). 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.

3.3. Mechanisms underlying male-biased expression of cntfa and pdlim3a in the medaka brain

In order to define the mechanisms underlying the male-biased expression of *cntfa* and *pdlim3a*, we first examined and compared the gene dosage of these genes in the male and female genomes (Fig. 3A and B). 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.

We 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 (Fig. 3C and D). 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.

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 (Fig. 3E). 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 (Fig. 3F). In females, *cntfa* expression, although not affected by ovariectomy, increased with E2 treatment; 11KT had no such effects (Fig. 3G). However, *pdlim3a* expression did not show clear responses to ovariectomy or subsequent treatment with 11KT or E2 (Fig. 3H).

4. Discussion

Here, we identified two gametologous genes with highly male-biased expression in the medaka brain by microarray profiling. By phylogenetic analysis, the genes were found to represent one of two teleost-specific paralogs encoding Cntf and Pdlim3, which presumably arose from a whole-genome duplication early in teleost evolution [21] and were designated *cntfa* and *pdlim3a*, respectively. The other paralogs (*cntfb* and *pdlim3b*) could not be found in the medaka genome; therefore, they were likely lost in this fish during evolution.

While previous studies identified several sex chromosome genes that were differentially expressed between the male and female brain in mammals and birds [3,4,6–10], this study is the first to demonstrate sexually dimorphic expression of sex chromosome genes in the brain of lower vertebrates. As opposed to the situation in mammals and birds, the sex chromosomes of lower 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 [17]. In light of this information, the location of *cntfa* and *pdlim3a* outside the sex-determining Y-specific region was somewhat unexpected.

This finding led us 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.

We 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

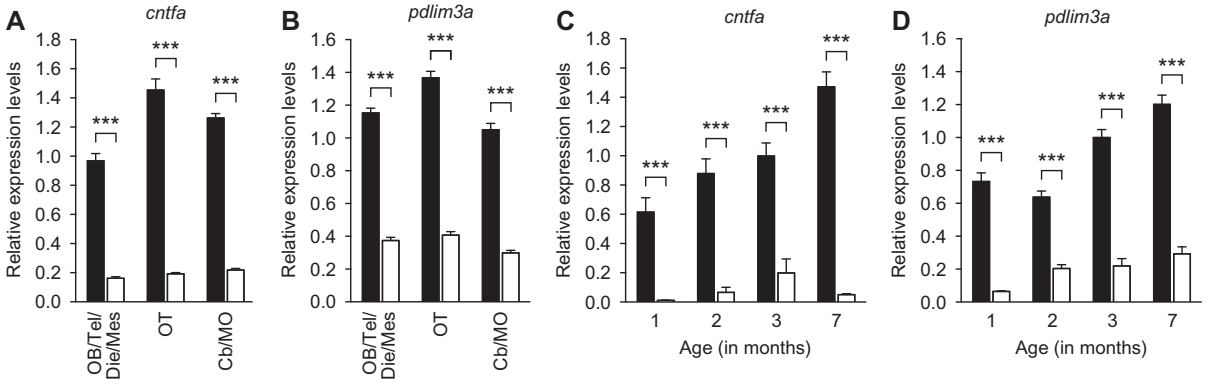

Fig. 2. Sex differences in the spatial and temporal expression of *cntfa* and *pdlim3a* in the medaka brain. (A and B) Sex differences in the levels of *cntfa* (A) and *pdlim3a* (B) expression in three portions of the brain: the olfactory bulb, telencephalon, diencephalon, and mesencephalon except the 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). (C and D) Sex differences in the levels of *cntfa* (C) and *pdlim3a* (D) expression 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 for both *cntfa* and *pdlim3a* ($p < 0.001$ for all). $^{***}p < 0.001$ between the sexes at the same age (Bonferroni's *post hoc* test).

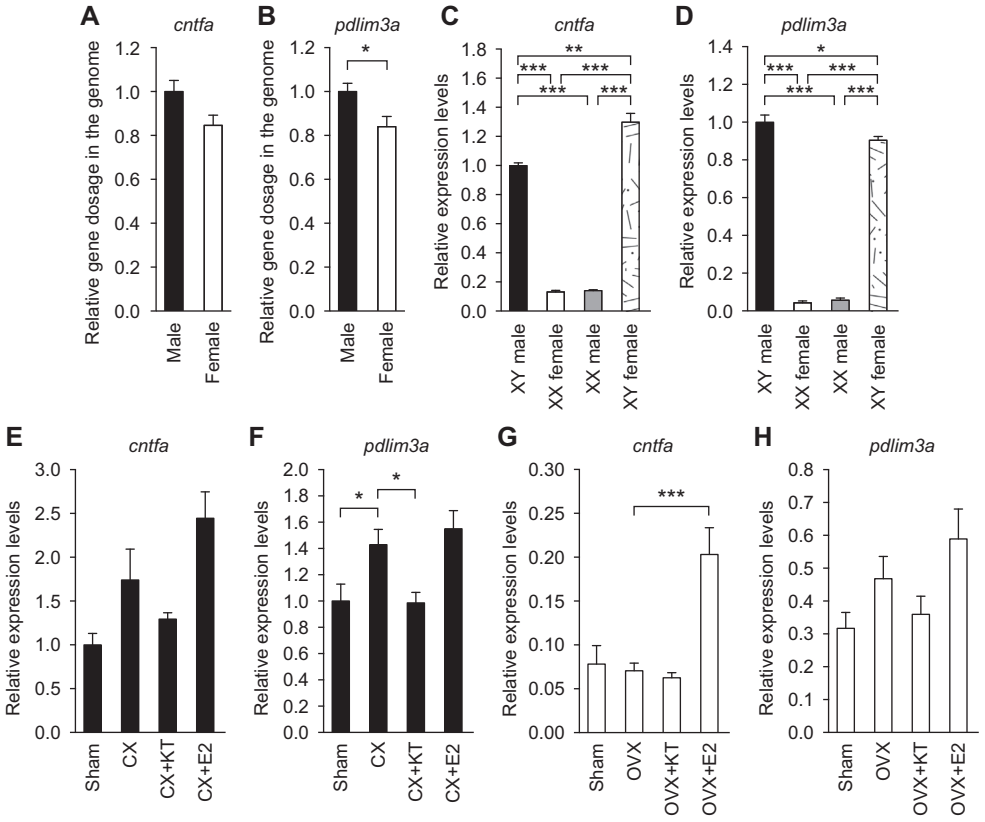

Fig. 3. Genetic and hormonal influences on *cntfa* and *pdlim3a* expression in the medaka brain. (A and B) Comparison of the gene dosage of *cntfa* (A) and *pdlim3a* (B) between the male and female genomes. $^{*}p < 0.05$ (unpaired *t*-test). (C and D) Expression of *cntfa* (C) and *pdlim3a* (D) in the brain of artificially sex-reversed medaka. The expression levels in sex-reversed XX males and XY 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). (E and F) The expression levels of *cntfa* (E) and *pdlim3a* (F) 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 estradiol-17β (E2) (CX + E2). $^{*}p < 0.05$ (Bonferroni's *post hoc* test). (G and H) The expression levels of *cntfa* (G) and *pdlim3a* (H) 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).

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) [17,22]. 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 [22]. In this context, our finding of the male-biased expression seems unique because the mutations on the Y chromosome would likely have activated the Y chromosome genes.

We 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. Our 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 [23]. 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. 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 is a polypeptide hormone belonging to the interleukin-6 family of cytokines. CNTF, which has been originally

described as a survival factor for neurons [24], 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 [25,26]. At present, little is known about Cntf in teleosts except that a *cntf*-like gene has been cloned in rainbow trout [27], which was found to be *cntfb*, one of the two *cntf* paralogs occurring in teleosts, by the phylogenetic analysis used in the present study. It may be of interest to note here that the teleost brain displays widespread neurogenesis throughout adulthood [28]. 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 100–1000-fold greater than that of mammals, because estrogen has the ability to stimulate neurogenesis [29,30]. Our 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 [31,32], the male-biased expression of *cntfa* may serve to reduce the female-biased acceleration of the cell life cycle in the medaka brain.

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 [33,34]. This protein was shown to be essential for the development of cardiac muscle [35]. 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 [36], no information is available about its role in 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 lower vertebrates, including teleost fish, have evolved simultaneously and independently in different genera or even species [16–18]. 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. Lower 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.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.131.

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