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ORIGINAL PAPER

Ontogenic expression patterns of several nuclear receptors and cytochrome P450 aromatases in brain and gonads of the Nile tilapia *Oreochromis niloticus* suggests their involvement in sex differentiation

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Abstract Using semi-quantitative reverse transcriptase polymerase chain reaction we analyzed the ontogenic expression patterns of several nuclear receptors (estrogen receptors [ERα and β], androgen receptors [ARα and β], Ad4BP/SF-1 and Dax-1) and cytochrome P450 aromatases (brain and ovarian types) in whole brain and gonads of the Nile tilapia. ERα and β transcripts were evident in both sexes with a high expression of ERα in females at 0 day after hatching (0 dah). ARα appeared early (0 dah) in males and while in females at 25 dah. Among the two types of cytochrome P450 aromatases, the expression of the brain type (bP450arom) but not the ovarian type (oP450arom) was evident from 0 to 90 dah in the whole brain of both males and females. Expression of Ad4BP/SF-1 in female brain began from 0 dah but in male brain at 5 dah. Expression of Dax-1 began at 0 dah and it was higher throughout in male brain than that of the female brain. In gonads, ERα and β transcripts were evident in both sexes with slight variation. In females, both oP450arom and Ad4BP/SF-1 amplicons were evident at 15 dah. In males, although faint expressions of Ad4BP/SF-1 amplicons were evident at early duration of development, oP450arom did not appear until 90 dah. Conversely, expression of bP450arom was observed throughout in the developing testis with varied pattern while in developing ovary it was evident till 15 dah and reappeared only after 90 dah. Taken together, present results suggest that brain acts merely as a synchronizer in the sex differentiation process initiated by gonadal cues/factors in the Nile tilapia.

Keywords Androgen receptor · Estrogen receptor · Ad4BP/SF-1 · Cytochrome P450 aromatases · Dax-1

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Introduction

Genetic, hormonal and environmental factors play a major role in the developmental organization of gonadal sex differentiation in teleosts (Nakamura et al. 1998; Nagahama 2000). Unlike gonadal sex differentiation, the mechanisms involved in sexual differentiation of the brain remain incompletely defined. Sexual differentiation of the brain is induced by gonadal steroids during the peri-natal critical period in most mammalian species (Maclusky and Naftolin 1981; Arnold and Gorsky 1984; Pilgrim and Hutchison 1994). In mammals and zebrafinch, it has been suggested that both estrogens and androgens play important roles during brain development by interfering with the maturation of distinct neural systems and, in particular, with the sexual differentiation of brain structures and function (Ivanova and Beyer 2000; Perlman et al. 2003).

In fish, sex steroids play a critical role to determine the sex and developmental processes even by overriding genetic sex determination (Yamamoto 1969; Nagahama 1994, 2000; Tsai et al. 2000; 2001). Administration of aromatase synthesis blockers favors the development of male while it is genetically prone for female sex (Nakamura et al. 1998). Similarly oral administration of methyl testosterone to genetic females resulted in male sex development (Nakamura et al. 1998). These studies designate sex steroids as prime factors to determine sex, however, whether it acts at the level of gonad and also at the level of brain is not yet been established. In mammals, testosterone secretion by the male during early development permanently alters the capacity of the brain to respond to circulating estrogen (McCarthy 1994). Perspective information from various studies indicates that gonadal factors determine sex differentiation or sex with synchronized cues from the factors of brain. There are no studies to understand such a contention in piscine species, as very few species have genetically controlled sex populations. The Nile tilapia is one of the species where genetically controlled male and female sex populations are available. In view of the lacunae in understanding brain sex differentiation, we analyzed the developmental expression patterns of nuclear steroid receptors such as the estrogen receptors (ERα and ERβ), androgen receptors (ARα and ARβ), cytochrome P450 aromatases (ovarian [oP450arom] and brain [bP450arom] types), Ad4BP/SF-1 and Dax-1 in brain and gonads of the Nile tilapia.

Materials and methods

The Nile tilapias (*Orechromis niloticus*) were reared in the laboratory in large tanks with a circulating aerated fresh water system. Fish (and fish fries) were fed ad libitum with commercial food pellets and maintained in natural light under constant temperature (26±1°C). In these conditions female tilapia spawns every two weeks (average of 14–18 days) where as the male remains in spermiating stage throughout the reproductive cycle after sexual maturation. All genetic females (XX) and males (XY) were obtained by artificial fertilization of eggs from normal females (XX) with milt obtained from either sex reversed (XX) or super males (YY), respectively. Brain and gonads were dissected out from fish at various developmental stages to study the expression pattern of various candidate cDNAs during ontogeny. The amplicons of ERα, ERβ, ARα, ARβ, oP450arom, bP450arom, Ad4BP/SF-1 and Dax-1 were analyzed by semi-quantitative reverse transcriptase polymerase chain reaction using gene specific primers (Table 1), in the developing brains of the Nile tilapia beginning from 0 day after hatching (dah) to pre-adult stage, in both the sexes. The developmental stages chosen were 0, 5, 10, 15, 20, 25, 40, 60, 75 and 90 dah. Similar stages were chosen to obtain gonads to perform RT-PCR for the correlates, however, at 0 and 5 dah whole body was used as dissection of gonads is tedious during these periods. Total RNA was extracted from whole brain and gonads (or whole body in the case of 0 and 5 dah) using Isogen solution (Wako, Osaka, Japan) according to the manufacturer's instructions. First strand cDNA were synthesized from 1 μg of total RNA with oligo-(dT)12–18 primers using Superscript-II RNAse H-Reverse transcriptase (GIBCO BRL, Rockville, MD, USA) according to manufacture's instructions. Later the first strand cDNA template was serially diluted to perform PCR at different cycles for different cDNAs. The semi-quantization was done by following the method of Kwon et al. (2001). Based on this quantification, optimal number of PCR cycles was

Table 1 Gene specific primers of various cDNAs tested in this study by RT-PCR

cDNA's name	Forward primer	Reverse primer	References
ARα	5′ GGAGACGCTCAGCTCCGCTTC 3′	5′ CCTCTTGAAGAACACCTTGCA 3′	Todo et al. (1999)
ARβ	5′ TCCACAAATCTTCCTTGTCTG 3′	5′ GGGCCTGGGTGGGAAGCTCAT 3′	Ikeuchi et al. (1999)
ERα	5′ GAAGGAAGCGTGCAATGAGC 3′	5′ CTCCATTGTGCCAGTGCAGA 3′	Chang et al. (1999)
ERβ	5′ TAACTGGACCAGCTGAGGGT 3′	5′ TCGCTGCAGTCTGAGGAACT 3′	Chang et al. (1999)
oP450arom	5′ GCTGAAGAACGGAAACTATACTTCAC 3′	5′ TGAAGCTGTCTCTCACCCACAACAGC 3′	Chang et al. (1997)
bP450arom	5′ TTTGAGGAGTGCCCACTACACCTCCAG 3′	5′ CACGTCTTTTTCATTCAAGATGTT 3′	Chang et al. (2005)
Ad4BP/SF-1	5′ GCTGTCTCATAACTGCTGGTC 3′	5′ TCTCGATCAGCAGGTTGTTG 3′	Yoshiura et al. (2003)
DAX-1	5′ GCTCTTCAACCCAGATCTGGAGGGT 3′	5′ CTACTTCCCGTAGAACATCTCCAT 3′	Wang et al. (2002)
β-Actin	5′ GGCATCACACCTTCTACAACGA 3′	5′ ACGCTCTGTCAGGATCTTCA 3′	Yoshiura et al. (2003)

determined as follows: 28 cycles for β-actin, between 30 and 32 cycles for different sex steroid receptors, oP450arom, bP450arom, Ad4BP/SF-1 and 36 cycles for Dax-1. The annealing temperature varied between 58 and 60°C depending on the primers for each gene. In general, PCR was performed with Amplitaq Gold DNA polymerase (Applied Biosystems, Foster city, CA, USA) for all samples using the gene specific primers for respective cDNAs (see Table 1). The following PCR conditions was used for amplification: 94°C 8 min 1 cycle; 94°C 1 min, 58–60°C 1 min 30 s, 72°C 2 min, 28–36 cycles (depending on the amplicons). PCR products were run on 1 or 1.5% ethidium bromide (EtBr) agarose gels. To verify the success of 1st strand cDNA synthesis, a β-actin control was used. The relative expression (band intensity analysis) of various amplicons to that of β-actin was calculated to compare the level of different transcripts between sexes and at different developmental stages. These data were shown in arbitrary units (Table 2) only for ERs and ARs in whole brain but not for other samples. Gonadal or brain tissues obtained from a single fry was completely insufficient to prepare either total RNA or first strand template, hence, a pool of gonadal or brain tissues obtained from group of fries were used in this study. No statistical comparisons were made since we used many fries (ranging from 25 to 30) to obtain two pools (for variability) of gonadal or brain samples to extract total RNA for first strand template preparation. We provided one representative RT-PCR photograph for each candidate gene analyzed.

Results

Expression of ERs and ARs in the whole brain of Nile tilapia during development

Amplicons of ERβ in male brain appeared quite late than that of the ERα with profound variation ERβ from 15 to 40 dah. Appearance of ARα amplicons in

Table 2 Ontogeny (expression pattern in arbitrary units) of both estrogen and androgen receptors in the brain of the Nile tilapia, *O. niloticus*

Days after hatching	ERα male brain	ERα female brain	ERβ male brain	ERβ female brain	ARα male brain	ARα female brain	ARβ male brain	ARβ female brain
0	Faint	+	Faint	V. Faint	+	Nil	Nil	+
5	+	+	+	V. Faint	+	Nil	+	++
10	+	+	+	V. Faint	+	Nil	+	++
15	+	+	++	+	++	Nil	+	++
20	+	+	+	+	+	Nil	+	++
25	+	+	Faint	+	+	+	+	+++
40	++	++	++	++	N.A.	N.A	N.A.	N.A
90	++	++	++	++	N.A.	N.A	N.A.	N.A
200 (Adult)	++	++	++	++	V. Faint	V. Faint	+	+

V. Faint, very faint; N.A., not analyzed

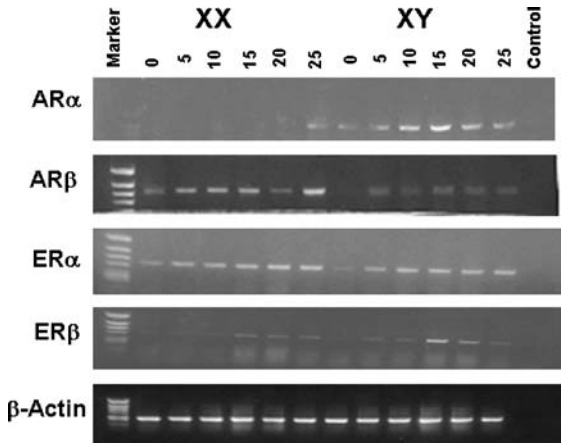

Fig. 1 RT-PCR analysis (representative gel) of expression patterns of androgen (ARα and ARβ) and estrogen (ERα and ERβ) receptors in the whole brain of Nile tilapia between 0 and 25 days after hatching (dah). Arabic numbers indicate the dah

brain showed minor variation based on the sex type. In females, ARα appeared only at 25 dah while its expression was evident in male brain from 0 dah onwards. On the other hand, ARβ amplicons were evident in both in male and female brains from 5 dah onwards. The expression patterns denoted in arbitrary units for ERs and ARs in the brain of Nile tilapia during the developmental period were tabulated in detail to show their minor differences between sexes (Table 2) till 200 dah (Fig. 1).

Expression of ERs in the gonads/whole body of Nile tilapia during development

Amplicons of ERα were evident in the gonads from 0 dah onwards. At 25 dah a faint expression is evident in developing ovary while no expression was evident in the developing testis. In the case of ERβ, the expression was evident in females till 25 dah but in the male the expression disappears at 25 dah. Although we studied the expression pattern of androgen receptors from 0 dah in the present study, we provided its expression pattern only at later durations (from 50 dah), as it was reported previously. Both ARα and β did not show variation in the gonads from 50 dah onwards. However, earlier durations showed profound variations between sexes (Ikeuchi et al. Unpublished data; present study) (Figs. 2 and 3).

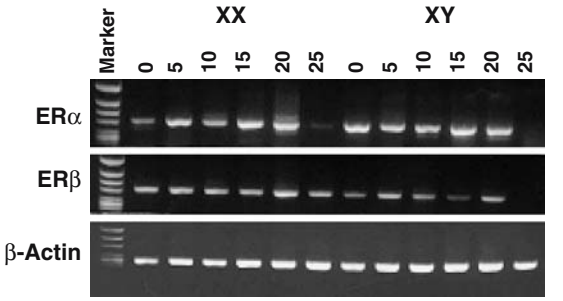

Fig. 2 RT-PCR analysis (representative gel) of expression patterns of estrogen (ERα and ERβ) receptors in the gonads/whole body of Nile tilapia between 0 and 25 dah. Whole body was used at 0 and 5 dah instead of gonads while 10 dah onwards (till 25 dah) gonads were used. Other details are as in Fig. 1

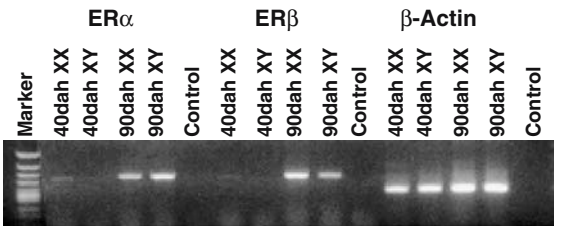

Fig. 3 RT-PCR analysis (representative gel) of expression patterns of estrogen (ERα and ERβ) receptors in the gonads of Nile tilapia at 40 and 90 dah. Other details are as in Fig. 1

Expressions of oP450arom, bP450arom, Ad4BP/SF-1 and Dax-1 in the whole brain of Nile tilapia during development

Amplicons of Ad4BP/SF-1 were more or less similar in male and female brains and it appeared from 0 dah (Fig. 4). Conversely, Dax-1 expression was intense only in male brain, however, measurable from 0 dah in both sexes (Fig. 5). In the Nile tilapia, amplicons of oP450arom were not at all evident in the developing brain tissues of either sex. On the other hand, bP450arom was consistently expressed in both male and female brains from day 0 to 90 dah. The initiation of expression of Ad4BP/SF-1 in the whole brain of females is from 0 dah but in males it appeared at 5 dah (Figs. 4 and 5).

Expression of oP450arom, bP450arom and Ad4BP/SF-1 in the gonads/whole body of Nile tilapia during development

Marked sexual dimorphism of oP450arom mRNA expression was observed. In females, the oP450arom

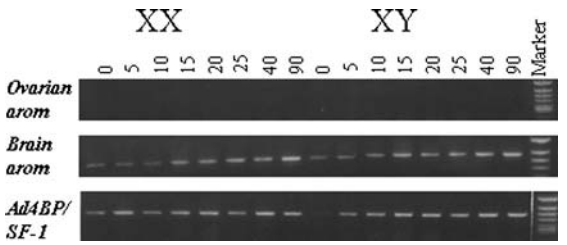

Fig. 4 RT-PCR analysis (representative gel) of expression patterns of ovarian type cytochrome P450 aromatase, brain type cytochrome P450 aromatase and Ad4BP/SF-1 in the whole brain of Nile tilapia between 0 and 90 dah. Other details are as in Fig. 1

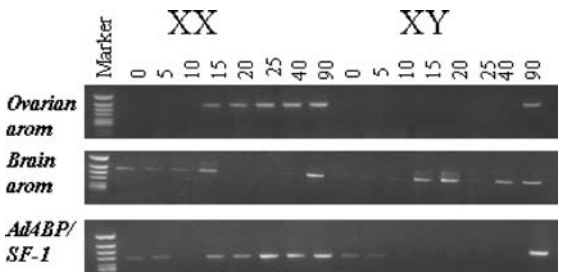

Fig. 5 RT-PCR analysis (representative gel) of expression patterns of ovarian type cytochrome P450 aromatase, brain type cytochrome P450 aromatase and Ad4BP/SF-1 in the gonads/whole body of Nile tilapia between 0 and 90 dah. Other details are as in Figs. 1 and 2

amplicons were evident at 15 dah while in males the expression is only observed at 90 dah. On the other hand, the bP450arom amplicons in the gonads during the early developmental period were identical in the beginning but after 15 dah developing ovary did not show any expression till 40 dah, yet, evident at 90 dah. In the developing testis, bP450arom expression was evident throughout while some stages showed faint expression. The expression pattern of Ad4BP/SF-1 in gonads showed synchrony with oP450arom. The expression pattern of Ad4BP/SF-1 was found to be evident in female gonads from 15 dah, while in males it appeared intensely from 90 dah onwards (Fig. 6).

Discussion

This report describes an integrated study of the expression patterns of several nuclear receptors and aromatases during the development of Nile tilapia.

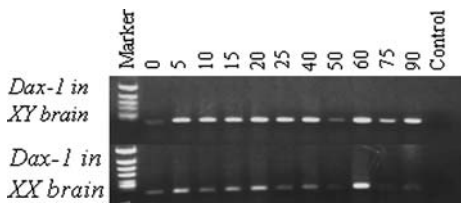

Fig. 6 RT-PCR analysis (representative gel) of expression patterns of Dax-1 in the whole brain of Nile tilapia between 0 and 90 dah. Other details are as in Fig. 1

We demonstrated the changes in the ontogenic expression patterns of ERs, ARs, cytochrome P450 aromatases, Ad4BP/SF-1 and Dax-1 in whole brain and/or gonads and attempted to correlate it with sexual development/differentiation. Tissue distribution pattern of these correlates also warrant for considering these genes as candidate markers for sex differentiation (Nagahama 2000). Among teleosts, ontogeny studies were carried out in zebrafish (Trant et al. 2001) and in the Nile tilapia (Kwon et al. 2001) for cytochrome P450 aromatases. However, these studies used whole body of the fries instead of gonadal or other tissues. It will be difficult to attribute more precise role if whole body is used instead of specific (gonadal) tissues. Hence, we dissected out the gonads from 10 dah onwards and the brains were separated even from 0 dah for the present investigation. We further intended to separate the regions of brain to evaluate their precise role, yet, the genes examined in the present study showed very poor expression individually and require tremendous pooling of tissues for quantification. These limited us to refrain from dividing the brain regions and used whole brain in the study. Despite this, present study is more comprehensive to explain the role of sex steroids in brain as well as gonadal sex differentiation.

In general, lack of sexual differences in the expression pattern of ARs and ERs in both brain and/or gonads (present study) indicate that the primordial gonad has the ability to develop either as testis or ovary depending on the endogenous hormone milieu or endocrine disruptors or oral hormone administration (Nagahama 2000). Thus, in teleosts, likewise in other higher vertebrates, the differentiation of testis or ovary is possibly from a bipotential gonadal primordium (Morrish and Sinclair 2002). Interestingly, ERα appears earlier than ERβ indicating the importance of former over the later. More recently in ewes it has been shown that ERα mediates seasonal

changes of estradiol in retrochaismatic area of hypothalamus (Hardy et al. 2003). The early appearance of ERα in Nile tilapia indicates its pivotal role and more precisely its expression appears to be intense in females, which might indicate some significance to it in female sex differentiation. Judging from germ cell multiplication, the development of male reproductive system is slower when compared to females (Nagahama 2000). This may also attribute the varied level of fluctuation in the expression patterns of ER and AR in male brain. Although the appearance of ARα is late (25 dah) in female brain (present study) and gonads (Todo et al. 1999), presence of ARβ (Ikeuchi et al. 1999; Ikeuchi et al. Unpublished data) might compensate the expression of ARα during sex reversal and development process. Perhaps similar expression patterns of sex steroid receptors might be one of the factors to create an irresolute tendency to the developing gonad. This clearly explains the action of (external) hormone-induced sex reversal process even before the differential expression of aromatase gene in presumptive gonadal area to produce endogenous hormone. Looking at this viewpoint, sex steroid receptors do play decisive roles in sex differentiation along with sex steroid hormones and its synthesizing enzymes. Furthermore, in the Nile tilapia, sex steroid receptors became prevalent even before the appearance of ligand(s). This is also evident from the appearance of ovarian P450 aromatase. However, present study needs validation by measuring the sex steroids.

Among the sex steroids, estrogens are considered important in fishes to determine the sex as estrogen synthesizing ability is achieved by the developing female gonad as early as 10–15 dah (Nakamura et al. 1998; Nagahama 2000). Any intervention to estrogen production for e.g. through oP450arom blockade resulted in the development of male sex while it is genetically prone to develop as female. Temporal expression pattern of oP450arom and Ad4BP/SF-1 in female gonads from 15 dah but not in male gonads support the concept that developing female has the ability to produce estrogens, which in turn might play a major role in sex differentiation. The functional significance of bP450arom in male gonad is not clear at present. In situ hybridization analysis from our laboratory indicated the presence of Ad4BP/SF-1 in both sexes; however, the level of expression is weak in males (Kobayshi et al. Unpublished data). Kobayashi et al. (2003) identified the expression of oP450 arom mRNA from 5 dah. Igiri et al. (manuscript in preparation) found that Ad4BP/SF-1 mRNA expresses in both XX and XY gonads 5–7 days after hatching. Perhaps the differences in the present study were due to high stringent conditions (annealing temperature 58 to 62°C) and whole body template which might have hampered the expression of Ad4BP/SF-1 (in males) and oP450arom (in females). Low expression of Ad4BP/SF-1 in male gonad indicates that the transcriptional up-regulation of bP450arom in male gonad might be different or rather lower as Ad4BP/SF-1 is considered to be one of the important transcriptional factors to promote aromatase expression. Lack of differences in these correlates in male and female brain indicates that gonadal factors play decisive role in sex differentiation. In accordance with our findings Kwon et al. (2001) also reported that bP450arom did not show any sexual dimorphism in brain. In addition, high brain aromatase content vis à vis estrogens in fish could be related to the continuous growth of their central nervous system during adulthood (Menuet et al. 2003). In the present study, intense expression of bP450arom in both sexes might indicate similar role in the developing brain of the Nile tilapia. Interestingly, oP450arom could not be detectable in the developing male and female brain. These results together suggest that the aromatases might play different roles based on their localization pattern during development, as variety of promoter elements were found in the aromatase genes (Yoshiura et al. 2003; Chang et al. 2005). The interaction of Ad4BP/SF-1and Dax-1 is well known in mammals (Kawabe et al. 1999). Although Ad4BP/SF-1 is considered as an important transcription factor for oP450arom (Yoshiura et al. 2003), the role of Dax-1 as a transcriptional factor for any steroidogenic enzyme genes is not yet clearly known in teleosts (Wang et al. 2002). Presence of high expression of Dax-1 in male brain indicates that Dax-1 might play a role in male brain sex differentiation. In summary, the results from this study propose that gonadal factors take the lead to determine the sex along with other important cues such as genetic and/or environmental factors. At least in the Nile tilapia, the role of brain is to merely act as a synchronizer for gonadal cues, which might be ultimately responsible to determine sex.

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References

Arnold AP, Gorsky RA (1984) Gonadal steroid induction of structural sex differences in the central nervous system. Ann Rev Neurosci 7:413–442

Chang XT, Kobayashi T, Senthilkumaran B, Kobayashi-Kajiura H, Sudhakumari CC, Nagahama Y (2005) Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol 141:101–115

Graves JAM (1998) Evolution of the mammalian Y chromosome and sex-determining genes. J Exp Zool 281:472–481

Hardy SL, Anderson GM, Valent M, Connors JM, Goodman RL (2003) Evidence that estrogen receptor α, but not β, mediates seasonal changes in the response of the ovine retrochiasmatic area to estradiol. Biol Reprod 68:846–852

Ikeuchi T, Todo T, Kobayashi T, Nagahama Y (1999) cDNA cloning of a novel androgen receptor subtype. J Biol Chem 274:25205–25209

Ivanova T, Beyer C (2000) Ontogenetic expression and sex differences of aromatase and estrogen receptor-α/β mRNA in the mouse hippocampus. Cell Tissue Res 300:231–237

Kawabe K, Shikayama T, Tsuboi H, Oka S, Oba K, Yanase T, Nawata H, Morohashi K (1999) Dax-1 as one of the target genes for Ad4BP/SF-1. Mol Endocrinol 13:1267–1284

Kobayashi T, Kaijura-Kobayashi and Y Nagahama 2003. Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, Tilapia. Cytogenet Genome Res. 102:289–294

Kwon JY, McAndrew BJ, Penman DJ (2001) Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia (*Oreochromis niloticus*). Mol Reprod Dev 59:359–370

MacLusky NJ, Naftolin F (1981) Sexual differentiation of the nervous system. Science 211:5925–5930

McCarthy MM (1994) Molecular aspects of sexual differentiation of the rodent brain. Psychoneuroendocrinology 19:415–427

Menuet A, Anglade I, Le Guevel R, Pellegrini E, Pakdel F, Kah O (2003) Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: comparison with estrogen receptor α. J Comp Neurol 462:180–193

Morrish BC, Sinclair AH (2002) Vertebrate sex determination: many means to an end. Reproduction 124:447–457

Nagahama Y (1994) Endocrine regulation of gametogenesis in fish. Int J Dev Biol 38:217–229

Nagahama Y (2000) Gonadal steroid hormones: major regulators of gonadal sex differentiation and gametogenesis in fish. In: Norberg O, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO (eds) Proc 6th Int Symp Rep Phy Fish. Bergen, Norway, pp 211–222

Nakamura M, Kobayashi T, Chang XT, Nagahama Y (1998) Gonadal sex differentiation in teleost fish. J Exp Zool 281:362–372

Pilgrim C, Hutchison JB (1994) Developmental regulation of sex differences in the brain: can the role of gonadal steroids redefined? Neuroscience 60:843–855

Perlman WR, Ramachandran B, Arnold AP (2003) Expression of androgen receptor mRNA in the late embryonic and early post hatch zebra finch brain. J Comp Neurol 455:513–530

Todo T, Ikeuchi T, Kobayashi T, Nagahama Y (1999) Fish androgen receptor: cDNA cloning, steroid activation of transcription in transfected mammalian cells, and tissue mRNA levels. Biochem Biophys Res Commun 254:378–383

Trant JM, Gavasso S, Ackers J, Chung BC, Place AR (2001) Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). J Exp Zool 290:475–483

Tsai CL, Wang LH, Chang CF, Kao CC (2000) Effects of gonadal steroids on brain serotonergic and aromatase activity during the critical period of sexual differentiation in tilapia, *Oreochromis mossambicus*. J Neuroendocrinol 12:894–898

Tsai CL, Wang LH, Fang LS (2001) Estradiol and para-cholorophenylalanine down regulate the expression of brain aromatase and estrogen receptor-alpha mRNA during their critical period of feminization in tilapia (*Oreochromis mossambicus*). Neuroendocrinology 74:325–334

Wang DS, Kobayashi T, Senthilkumaran B, Sakai F, Sudhakumari CC, Suzuki T, Yoshikuni M, Matsuda M, Morohashi K, Nagahama Y (2002) Molecular cloning of DAX1 and SHP cDNAs and their expression patterns in the Nile tilapia, *Oreochromis niloticus*. Biochem Biophys Res Commun 297:632–640

Yamamoto T (1969) Sex differentiation. In: Hoar WS, Randall DJ (eds) Fish physiology, vol III. Academic Press, New York, pp 117–175

Yoshiura Y, Senthilkumaran B, Watanabe M, Oba Y, Kobayshi T, Nagahama Y (2003) Synergistic expression of Ad4BP/SF-1 and cytochrome P450 aromatse (ovarian type) in the ovary of Nile tilapia, *Oreochromis niloticus*, during vitellogenesis suggests transcriptional interaction. Biol Reprod 68:1545–1553