Female-specific target sites for both oestrogen and androgen in the teleost brain

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To dissect the molecular and cellular basis of sexual differentiation of the teleost brain, which maintains marked sexual plasticity throughout life, we examined sex differences in neural expression of all subtypes of nuclear oestrogen and androgen receptors (ER and AR) in medaka. All receptors were differentially expressed between the sexes in specific nuclei in the forebrain. The most pronounced sex differences were found in several nuclei in the ventral telencephalic and preoptic areas, where ER and AR expression were prominent in females but almost completely absent in males, indicating that these nuclei represent female-specific target sites for both oestrogen and androgen in the brain. Subsequent analyses revealed that the female-specific expression of ER and AR is not under the direct control of sex-linked genes but is instead regulated positively by oestrogen and negatively by androgen in a transient and reversible manner. Taken together, the present study demonstrates that sex-specific target sites for both oestrogen and androgen occur in the brain as a result of the activational effects of gonadal steroids. The consequent sex-specific but reversible steroid sensitivity of the adult brain probably contributes substantially to the process of sexual differentiation and the persistent sexual plasticity of the teleost brain.

Keywords: teleost; brain; sex differences; sexual plasticity; oestrogen receptor; androgen receptor

1. INTRODUCTION

Oestrogen and androgen play critical roles in sexual differentiation of the vertebrate brain [1]. These sex steroid hormones regulate a wide range of physiological and behavioural traits (including neuroendocrine hormone secretion, reproductive behaviour and aggression) in a sex-dependent manner. Most of these effects depend on activation of nuclear oestrogen and androgen receptors (ER and AR) expressed in the brain. Because there are pronounced sex differences in the effectiveness of oestrogen and androgen on some of these traits, ER and AR are presumably expressed in a sex-dependent manner in the brain. Indeed, sex differences in their expression that could underlie the differences in traits have been identified in several brain nuclei of many vertebrate species [2–4].

In teleost fishes, however, there have been no studies demonstrating sex differences in the expression of ER and AR in brain nuclei. The teleost brain is unique in that it exhibits a considerable degree of sexual plasticity throughout the lifetime of the fish [5]. Although the mammalian brain is also initially plastic, and equally capable of assuming a male or female phenotype, it undergoes an irreversible process of sexual differentiation during perinatal development. Conversely, the phenotypic sex of teleosts, including sex-specific reproductive behaviour, can be manipulated by exposure to exogenous oestrogen or androgen, even after sexual maturity [6]. Furthermore, a large number of teleost species spontaneously undergo phenotypic sex inversion, even in adulthood [5]. These phenomena indicate that teleosts must have distinctive mechanisms of sexual differentiation of the brain that remain largely unknown. For these reasons, the teleost is a good model for providing insights into the mechanisms underlying the sexual plasticity of the brain and the evolutionary processes in the sexual differentiation of the vertebrate brain.

Medaka (Oryzias latipes) offer a number of advantages as a teleost model for sex differences and sexual differentiation. For example, medaka represent one of a few teleost species in which the sex-determining gene has been identified [7,8]. Furthermore, its phenotypic sex can easily be manipulated by hormonal treatment [9]. Teleosts, including medaka, have three paralogous genes for ER—esr1 (or ERα), esr2a (or ERβ1) and esr2b (or ERβ2)—and two genes for AR—ara (or ARαs) and arb (or ARβ), which arose from the whole genome duplication early in teleost evolution [10,11]. Here, we assessed the expression of these genes in the medaka brain and found that several brain nuclei express both ER and AR almost exclusively only in females, representing female-specific target sites for both oestrogen and

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androgen. Subsequent analyses revealed that the female-specific expression of ER and AR in these brain nuclei arises from the activational effects of gonadal steroids, and is therefore sexually plastic even in adulthood, thereby perhaps contributing to the remarkable sexual plasticity of the teleost brain.

2. MATERIAL AND METHODS

(a) Animals

The use and care of animals in this study were performed in accordance with the guidelines of the Committee on Life Sciences of the University of Tokyo. Medaka of the d-rR strain were bred and maintained at 28°C with a 14 L : 10 D photoperiod, and were fed three to four times a day with live brine shrimp and commercial pellet food. Sexually mature spawning adult fish of 3 to 5 months post-fertilization (mpf) were sampled at 1–2.5 h following the onset of light and used for analyses, unless otherwise noted.

(b) Examination of sex differences in the overall expression of ER and AR in the brain

Sex differences in the overall expression of ER and AR in the medaka brain were examined throughout the diurnal/reproductive cycles by real-time PCR (medaka undergo daily cycles of gametogenesis and spawning; they typically spawn around the time the lights come on at 9.00). The whole brain was removed from sexually mature medaka of both sexes at 4 h intervals across a 24 h period (6.00, 10.00, 14.00, 18.00, 22.00 and 2.00; n = 7 for each sex at each sampling time point). Total RNA was isolated from the brain using RNaseasy lipid tissue mini kit (Qiagen, Hilden, Germany) with DNase treatment (Qiagen). cDNA was synthesized using Omniscript RT kit (Qiagen) supplemented with random nonamers (Takara Bio, Shiga, Japan). Real-time PCR was performed using LightCycler 480 System II with LightCycler 480 SYBR Green I Master (Roche Diagnostics). Whole brains dissected from sexually mature medaka of both sexes at 4 h intervals were used for real-time PCR analyses, unless otherwise noted.

(c) Identification of brain nuclei responsible for sex differences in ER and AR expression

The DNA fragments corresponding to nucleotides 1672–2759, 1838–3039, 1828–3037, 53–1233 and 16–503 of the esr1 (D28954), esr2a (AB070901, together with the genome database), esr2b (AB428449, together with the genome database), ara (AB076399) and arb (EU100398) cDNAs, respectively, were PCR-amplified and used to generate digoxigenin (DIG)-labelled cRNA probes, with the DIG RNA labelling mix (Roche Diagnostics). Whole brains dissected from sexually mature medaka of both sexes (n = 4) were fixed in 4 per cent paraformaldehyde (PFA) for 6–7 h, dehydrated in ethanol and embedded in paraffin. The sections were digested with proteinase K (Wako Pure Chemical Industries, Osaka, Japan) for 15 min at 37°C, postfixed with 4 per cent PFA for 10 min, and acetylated with 0.25 per cent acetic anhydride in 0.1 M triethanolamine for 15 min. Hybridization was conducted overnight at 55°C with the earlier-described DIG-labelled probes in hybridization buffer: 50 per cent formamide, 5x saline-sodium citrate (SSC) 5x Denhardt’s solution, 2 mg ml⁻¹ yeast RNA and 30 μg ml⁻¹ calf thymus DNA. The sections were washed in 5x SSC, 25 per cent formamide for 20 min at 55°C and in 2x SSC for 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 : 2000–5000 and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Roche Diagnostics), following the manufacturer’s instructions. The colour was allowed to develop overnight in the dark. For semi-quantitative analysis, all sections throughout the brain were photographed and converted to black and white binary images by thresholding using PHOTOSHOP (Adobe Systems, San Jose, CA). The total area of ER and AR expression was calculated for each nucleus using ImageJ (http://rsweb.nih.gov/ij/). The subdivisions and nomenclature of brain nuclei were taken from the medaka brain atlases [12,13] (http://www.shigen.nig.ac.jp/medaka/medaka_atlas/) and extensively supplemented with information obtained from our Nissl-stained sections.

(d) Examination of genetic and phenotypic sex dependence of ER and AR expression

Sex-inverted medaka were produced as described previously [14]. Briefly, fertilized eggs were incubated at high temperature (32°C) and simultaneously received 0.2 ng ml⁻¹ methyltestosterone until hatching, which led to the production of XX males. XY female fish were obtained by exposing fertilized eggs to 200 ng ml⁻¹ 17β-oestradiol (E2) until hatching. Whole brains were removed from XX males and XY females, as well as wild-type XY males and XX females, all of which were sexually mature (n = 8 for each group). Real-time PCR was carried out to assess ER and AR expression as described earlier.

(e) Evaluation of the effects of gonadal sex steroids on ER and AR expression

The ovary was surgically removed from sexually mature females following the procedure from an early study [15]. Ovariectomized fish were treated with 100 ng ml⁻¹ of E2 or 11-ketotestosterone (11-KT; a prominent, non-aromatizable teleost androgen) or vehicle alone (ethanol) by immersion for 5 days (n = 10 for each group). Sham-operated female fish (n = 12) were treated with vehicle alone as controls. The whole brains of these fish were dissected out and used for real-time PCR to assess the effects of gonadal sex steroids on the overall expression of ER and AR in the brain as described earlier. Semi-quantitative in situ hybridization was also carried out using the whole brain (n = 8 for each group) as described earlier, to appreciate the steroidal effects on ER and AR expression in respective brain nuclei where they were differentially expressed between the sexes.

(f) Statistical analysis

In all real-time PCR analyses, the expression levels of ER and AR (normalized by that of actb) in sexually mature male brain were arbitrarily set to one, and the relative difference was calculated, in order to facilitate comparisons among analyses. Statistical analyses were performed using Prism software (GraphPad Software, San Diego, CA). Comparisons

The expression of ER and AR could vary depending on the diurnal and/or reproductive cycle, and, accordingly, was assessed throughout the day (see the electronic supplementary material, figure S1). Higher levels of *esr1* was assessed throughout the day (see the electronic supplementary material, figure S2). While no sex differences were detected for *esr1* expression through the spawning period, *esr2a* began to exhibit significantly higher expression in the male brain than the female brain during the late spawning period (7 mpf). By contrast, *esr2b* exhibited greater levels of expression in the female brain than in the male brain as early as at the onset of secondary sexual characteristics (1 mpf), and the difference between sexes increased with sexual maturation. The gene *ara* exhibited significantly higher expression in females only during the early spawning period (3 mpf), while significant female-biased expression of *arb* first appeared at the onset of puberty (2 mpf), and persisted during the spawning period.

**3. RESULTS**

(a) **Sex differences in the overall expression of ER and AR in the brain**

We first examined the occurrence of sex differences in the overall ER and AR expression in the adult medaka brain. The expression of ER and AR could vary depending on the diurnal and/or reproductive cycle, and, accordingly, was assessed throughout the day (see the electronic supplementary material, figure S1). Higher levels of *esr1* expression were observed in the male brain at all time points examined except at 10.00. While no significant sex differences in *esr2a* expression were detected at any time point examined, *esr2b* was consistently expressed at higher levels in the female brain, except at 22.00. A significant female bias was also apparent in *ara* expression at 10.00, and in *arb* expression at 10.00, 14.00 and 2.00.

Subsequently, developmental changes in the overall expression of ER and AR in the medaka brain were investigated to determine when, during sexual maturation, the expression was sexually differentiated (see the electronic supplementary material, figure S2). While no sex differences were detected for *esr1* expression through the spawning period, *esr2a* began to exhibit significantly higher expression in the male brain than the female brain during the late spawning period (7 mpf). By contrast, *esr2b* exhibited greater levels of expression in the female brain than in the male brain as early as at the onset of secondary sexual characteristics (1 mpf), and the difference between sexes increased with sexual maturation. The gene *ara* exhibited significantly higher expression in females only during the early spawning period (3 mpf), while significant female-biased expression of *arb* first appeared at the onset of puberty (2 mpf), and persisted during the spawning period.

(b) **Identification of brain nuclei responsible for sex differences in ER and AR expression**

We then determined the brain nuclei that were the sources of ER (figure 1) and AR (figure 2) expression and their sex differences in sexually mature medaka. Abbreviations for medaka brain nuclei are given in table 1. Some genes were continuously expressed over two or more nuclei in certain brain regions, where it was difficult to analyse expression separately in each nucleus.
Table 1. Abbreviations of the brain nucleus.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aPPp</td>
<td>anterior part of posterior parvocellular preoptic nucleus</td>
</tr>
<tr>
<td>IQ</td>
<td>inferior oblique of the nucleus of oculomotor nerve</td>
</tr>
<tr>
<td>NAT</td>
<td>nucleus anterior tuberis</td>
</tr>
<tr>
<td>NPT</td>
<td>nucleus posterior tuberis</td>
</tr>
<tr>
<td>NRP</td>
<td>posterior recess nucleus</td>
</tr>
<tr>
<td>NVT</td>
<td>nucleus ventral tuberis</td>
</tr>
<tr>
<td>PGZ3</td>
<td>periventricular grey zone (layer 3)</td>
</tr>
<tr>
<td>PMm</td>
<td>magnocellular portion of the magnocellular preoptic nucleus</td>
</tr>
<tr>
<td>PMp</td>
<td>parvocellular portion of the magnocellular preoptic nucleus</td>
</tr>
<tr>
<td>PPa</td>
<td>anterior parvocellular preoptic nucleus</td>
</tr>
<tr>
<td>pPPp</td>
<td>posterior part of posterior parvocellular preoptic nucleus</td>
</tr>
<tr>
<td>RT</td>
<td>rostral tegmental nucleus</td>
</tr>
<tr>
<td>Vd</td>
<td>dorsal nucleus of the ventral telencephalic area</td>
</tr>
<tr>
<td>VM</td>
<td>ventromedial nucleus (thalamus)</td>
</tr>
<tr>
<td>Vp</td>
<td>posterior nucleus of the ventral telencephalic area</td>
</tr>
<tr>
<td>Vs</td>
<td>supracommissural nucleus of the ventral telencephalic area</td>
</tr>
<tr>
<td>Vv</td>
<td>ventral nucleus of the ventral telencephalic area</td>
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In this case, the total area of expression was summed over the nuclei, and the names of the nuclei are separated by slashes.

The gene esr1 was expressed in the posterior nucleus of the ventral telencephalic area (Vp) in the telencephalon; the parvocellular portion of the magnocellular preoptic nucleus (PMp) and the anterior part of the posterior parvocellular preoptic nucleus (aPPp) in the preoptic area; and the nuclei ventral tuberis (NVT) and posterior tuberis (NPT) in the hypothalamus (figure 1). Its expression in aPPp was almost specific to females; none to a few esr1-expressing neurons were detected in males. On the other hand, esr2a was expressed in the ventral nucleus of the ventral telencephalic area (Vv) and the dorsal/supracommissural/posterior nuclei of the ventral telencephalic area (Vd/Vs/Vp) in the telencephalon; PMp and the anterior parvocellular preoptic nucleus (PPa) in the preoptic area; the posterior part of the posterior parvocellular preoptic nucleus (pPPp) in the thalamus; and NVT/the posterior recess nucleus (NRP) in the hypothalamus (figure 1). All nuclei in the preoptic area, thalamus and hypothalamus had a significantly higher expression of esr2a in males than in females. The gene esr2b was expressed in Vs/Vp in the telencephalon; PMp, PPa and the magnocellular/gigantocellular portions of the magnocellular preoptic nucleus (PMm/PMg)/aPPp in the preoptic area; pPPp and the ventromedial nucleus (VM) in the thalamus; the nucleus anterior tuberis (NAT), NVT and NPT in the hypothalamus; and the inferior oblique of the nucleus of oculomotor nerve (IQ)/rostral tegmental nucleus (RT) in the tegmentum (figure 1). esr2b expression in Vs/Vp, PMm/PMg/aPPp and pPPp was almost entirely female-specific; males had none to a few esr2b-expressing neurons in these nuclei.

Neurons expressing ara were observed in Vv and Vs/Vp in the telencephalon; PMp, PPa and aPPp in the preoptic area; pPPp in the thalamus; NVT and NPT in the
hypothalamus; and the periventricular grey zone (layer 3; PGZ3) in the optic tectum (figure 2). Males had a significantly greater expression of *ara* in NPT. The gene *arb* was expressed in Vs/Vp in the telencephalon; PMP, PPa and PMM/PMG/aPPp in the preoptic area; pPPp and VM in the thalamus; and NAT, NVT and NPT in the hypothalamus; and PGZ3 in the optic tectum (figure 2). All nuclei in the telencephalon, preoptic area and hypothalamus, except NVT, contained more *arb* expression in females than males. The most striking sex differences were found in Vs/Vp, PMM/PMG/aPPp and NPT, where *arb* expression was restricted almost exclusively to females.

The distribution of sexually dimorphic expression of ER and AR in the medaka brain is illustrated in figure 3. Vs/Vp and PMM/PMG/aPPp exhibited the most conspicuous sex difference, where both ER and AR were predominantly expressed in females but were almost completely absent in males.

(c) Genetic and phenotypic sex dependence of ER and AR expression

Next, in order to understand the magnitude of genetic and hormonal influences on the mechanisms underlying sexual differentiation in the neural expression of ER and AR, we produced sex-inverted fish to investigate if sex-dependent expression patterns coincided with genetic sex or phenotypic sex (see the electronic supplementary material, figure S3). XY females exhibited the same

high level of neural expression of *esr2b*, *ara* and *arb* as wild-type XX females, whereas XX males expressed these genes at a lower level, comparable to wild-type XY males. There were no significant differences in *esr1* and *esr2a* expression between XX males and XY females.

(d) Effects of gonadal sex steroids on ER and AR expression

We subsequently examined the effects of gonadal steroids on the overall ER and AR expression in the brain by using females subjected to sham operation, ovariectomy or ovariectomy plus E2 or 11-KT treatment (see the electronic supplementary material, figure S4). There were no statistically significant differences between sham-operated and ovariectomized fish in the overall expression of any of the genes. E2 treatment, however, led to a significant increase in the expression of all genes except *esr2a* compared with the ovariectomized fish. By contrast, 11-KT significantly decreased the expression of *esr2b* and *arb*.

We then evaluated the effects of gonadal steroids on the expression of ER and AR in the respective brain nuclei where they were differentially expressed between the sexes, by means of an ovariectomy, followed by steroid treatment as described earlier. The results of the female-biased expression of ER and AR are shown in figures 4 and 5, respectively. The results of the male-biased ER and AR expression are shown in electronic supplementary material, figures S5 and S6, respectively. Ovariectomy caused a
expression of testosterone [22]. Meanwhile, brain aromatase activity in females undergo a large preovulatory surge of circulating testosterone sometimes equal to the levels found in males; females lating testosterone levels in females are substantial and are between the sexes in the medaka brain. In teleosts, circulating aromatase activity is known about its sex differences, with only one study investigating AR expression levels in the whole brain of the wrasse [21]. In the present study, we demonstrated that expression in the brain of teleosts [16–20], virtually noth-

While several studies have addressed ER and AR expression in brain sexual differentiation in teleosts are distinct from those in other vertebrates. For instance, as opposed to the situation in the mammalian and avian brain, in which Estrl is expressed more intensely in females in several nuclei [2,24,25], the female-biased expression of estrl occurs only in a nucleus in medaka, and males show greater overall estrl expression in the medaka brain. This observation implies a unique role for the brain estrl in neural masculinization or defeminization. In addition, ar and ar are expressed at approximately equal levels between the sexes and at higher levels in females, respectively, in the medaka brain; this is in contrast to the situation in mammals, where Ar is generally expressed to a greater extent in the male brain than in the female brain, thus playing a critical role in the masculinization of reproductive tissues is 100–1000 times greater than in mammals and birds, resulting in a high amount of oestrogen even in the male brain [23]. Considering these facts, sex differences in the brain steroid milieu in the teleost brain are likely to be smaller compared with other vertebrates. Our present observation of the marked sexual dimorphism in neural ER and AR expression suggests that sex differences in the central action of oestrogen and androgen are primarily controlled at the receptor level rather than at the ligand level. Sex differences in the sensitivity of the brain to oestrogen and androgen are thus the most likely underlying mechanism for brain sexual differentiation in teleosts, at least in medaka.

Intriguingly, the patterns of sex differences in central ER and AR expression in medaka are very different from those observed in other vertebrates, suggesting that roles for ER and AR in brain sexual differentiation in teleosts are distinct from those in other vertebrates. For instance, as opposed to the situation in the mammalian and avian brain, in which Estrl is expressed more intensely in females in several nuclei [2,24,25], the female-biased expression of estrl occurs only in a nucleus in medaka, and males show greater overall estrl expression in the medaka brain. This observation implies a unique role for the brain estrl in neural masculinization or feminization. In addition, ar and ar are expressed at approximately equal levels between the sexes and at higher levels in females, respectively, in the medaka brain; this is in contrast to the situation in mammals, where Ar is generally expressed to a greater extent in the male brain than in the female brain, thus playing a critical role in the masculinization of reproductive tissues is 100–1000 times greater than in mammals and birds, resulting in a high amount of oestrogen even in the male brain [23]. Considering these facts, sex differences in the brain steroid milieu in the teleost brain are likely to be smaller compared with other vertebrates. Our present observation of the marked sexual dimorphism in neural ER and AR expression suggests that sex differences in the central action of oestrogen and androgen are primarily controlled at the receptor level rather than at the ligand level. Sex differences in the sensitivity of the brain to oestrogen and androgen are thus the most likely underlying mechanism for brain sexual differentiation in teleosts, at least in medaka.

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behaviour [26–29]. These results suggest that androgen/AR signalling has some relevance to feminization or demasculinization of the female brain in teleosts.

It should be noted that there are pronounced sex differences in ER and/or AR expression in several nuclei of the medaka brain, where their expression is prominent in one sex but nearly or fully absent in the other sex. Most surprisingly, PMm/PMg/aPPp in the preoptic area abundantly express esr1, esr2b and arb in females, but there is little to no expression of any of the receptors in males. A similar situation occurs in Vs/Vp in the telencephalon, where, although both males and females more or less express all the receptor genes, the expression of the most dominant receptors in these nuclei (esr2b and arb) is almost completely specific to females. This finding indicates that these nuclei represent female-specific target sites for both oestrogen and androgen in the medaka brain. To the best of our knowledge, this is the first report of the existence of sex-specific target nuclei for both oestrogen and androgen in the brain.

PMm/PMg consist of small populations of large neurosecretory neurons, including isotocin- and vasotocin-producing neurons [30]. aPPp lies immediately ventral to PMm/PMg and contains a relatively large population of small neurosecretory neurons. Although still controversial, aPPp is assumed to be homologous to the paraventricular nucleus or medial preoptic area in mammals [31,32]. Vs/Vp are considered to be homologous to the bed nucleus of stria terminalis (BNST) in mammals [33,34]. The principal nucleus of the BNST in mammals is larger and contains more neurons in males than females, and plays an important role in male reproductive behaviour [35–37]. Consistent with these lines of evidence, males harbour more intense expression of Esr2 and Ar than females in this nucleus in mammals [27]. Our present results revealed that, as opposed to the mammalian BNST, Vs/Vp as well as PMm/PMg/aPPp in medaka express ER and AR almost specifically in females. Therefore, these brain nuclei are likely to be receptive to oestrogen and androgen only in females, and relevant to female-specific brain function induced by oestrogen and androgen. It is of interest to note that each of PMm/PMg/aPPp and Vs/Vp in teleosts has been implicated, by classic lesion and electrical stimulation studies, in reproductive behaviour [38–41]. It is thus reasonable to assume that the female-specific expression of ER and AR in these brain nuclei in teleosts is responsible for promoting female-typical or suppressing male-typical reproductive behaviour in females. Consistent with this idea, we found here that in the medaka brain,
esr2b, ara and arb have the largest female bias in overall expression around the onset of the light phase, when medaka usually spawn, further suggesting their involvement in reproductive behaviour.

The question then arises as to the mechanisms that underlie the sexually dimorphic expression of ER and AR in the medaka brain. Accumulating evidence suggests that some parts of the mammalian and avian brains sexually differentiate according to a cell-autonomous genetic programme determined by genes on the sex chromosome, referred to as ‘sex chromosome’ effects [42–44]. In the present study, we evaluated the possible involvement of sex chromosome genes in sex-dependent ER and AR expression in the medaka brain by generating and analysing sex-inverted fish. The overall expression levels of ER and AR in the medaka brain were correlated not with genetic sex but rather with phenotypic sex, indicating that their sex differences do not arise from the sex chromosome effects. Instead, the result implies that hormonal factors are the root cause of sex differences in the expression of ER and AR in the medaka brain.

Accordingly, we then assessed the effects of gonadal steroids on the sexually dimorphic expression of ER and AR, and demonstrated that their expression is indeed significantly influenced by gonadal steroids. Notably, in the brain nuclei where females exhibit higher expression of ER (esr1 and esr2b), these genes are regulated positively by gonadal E2 and negatively by 11-KT, the primary androgen in teleosts, thus being consistent with the female-specific expression in these nuclei. In marked contrast to the current observation, the suppressive effects of oestrogen on Esr1 and Esr2 expression have been repeatedly described in the mammalian brain [45], suggesting different oestrogenic regulatory systems for central ER expression in teleosts and mammals. The inhibitory effect of androgen on the medaka ER is probably mediated by AR, as 11-KT is a non- aromatizable androgen. Hence, the female-specific ER expression in the medaka brain arises through the combination of oestrogen upregulation via ER itself and androgen downregulation via AR. The other ER form in medaka brain, esr2a, shows no clear responses to any hormonal alterations, although it exhibits robust male-biased expression in several brain nuclei. The regulatory mechanisms for esr2a consequently remain unknown. Our data reveal that the expression of both ara and arb is also regulated positively by gonadal E2. In addition, the overall expression of arb in the brain is influenced negatively by 11-KT, although this effect is significant only in Vs/Vp at the level of the nucleus and thus appears somewhat modest. The cross-talk between oestrogen/ER and androgen/AR signalling probably underlies AR expression in the medaka brain as well. Importantly, these regulatory mechanisms for ER and AR expression involving the self-feedback cycling of ligand–receptor events, as well as the cross-talk of the signalling pathways, have a potential to synergistically enhance or, conversely, abolish the central action of oestrogen and androgen following even slight changes in the steroid milieu. This should be an efficient system for causing sexual differentiation of the teleost brain, where the steroid milieu often appears to differ relatively little between the sexes.

Another important aspect of the present findings is the reversibility of sex-dependent expression patterns of ER and AR in the brain. The robust expression of ER and AR in females is diminished to the same low level as in males by ovariectomy and androgen treatment, indicating that the female-typical expression patterns could be converted almost completely to those seen in males, under two conditions: without a constant supply of oestrogen from the ovary or with an increase in the circulating androgen. The sexually dimorphic expression of ER and AR in the medaka brain thus depends largely (or even perhaps solely) on the transient and reversible effects of adult gonadal steroids, which should be regarded as ‘activational’ effects, unlike in the mammalian brain, where Esr1 and Esr2 expression is epigenetically programmed in a permanent and irreversible manner by the ‘organizational’ effects of sex steroids during the perinatal period [46,47]. This view is further supported by the observation that sex differences of ER and AR expression in the medaka brain appear concomitantly with the onset of sexual maturation. The consequence of full reversibility of the sex-specific neural ER and AR expression patterns is that sexually differentiated sensitivity of respective brain nuclei to sex steroids, which defines the site and degree of steroid action differentially in the male and female brain, can be inverted between the sexes. The considerable and enduring sexual plasticity of the teleost brain is presumably attributable to this reversible sexual dimorphism in neural sensitivity to sex steroids. Similar activational effects of sex steroids and sexual reversibility have been observed for the expression of aromatase in the brain of several teleost species [14,48–50]. This steroidogenic enzyme, as well as sex steroid receptors, may contribute substantially to determine the sexual phenotype of the teleost brain by modulating the balance between oestrogen and androgen signalling in the brain.

In conclusion, we found that the expression of both ER and AR in several telencephalic and preoptic nuclei of the medaka brain is prominent in females but almost completely absent in males, indicating that these nuclei represent female-specific target sites for both oestrogen and androgen. Most of the sexually dimorphic expression of ER and AR in the medaka brain, including that in the female-specific steroid target nuclei, arises largely, if not entirely, from the activational effects of oestrogen and androgen, and is therefore sexually plastic, even in adulthood. The resultant sex-specific, but reversible, sensitivity to oestrogen and androgen in the adult brain probably contributes significantly to the process of sexual differentiation and a remarkable degree of sexual plasticity in the teleost brain.

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