Sexual Dimorphism of Brain Aromatase Activity in Medaka: Induction of a Female Phenotype by Estradiol

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In this study we identified sex-dependent dimorphism of brain aromatase in the teleost medaka and examined its regulation by sex steriods. We first investigated differential distribution of brain aromatase activity in sexually mature male and female medaka in serial coronal sections of the brain and identified the hypothalamic nuclei contained in each section using the brain atlas of medaka. In the brain of male medaka, high levels of activity are localized in sections containing the preoptic (POA) and suprachiasmatic nuclei (SC) (63–75 fmol/hr) and low levels in the nuclei periventricular dorsalis (HD), ventralis (HV), and caudalis (Hc), nuclei diffusus of lobulus inferiores (NDIL), and nuclei tuberi anteriores (TA) and posteriores (TP) (< 25 fmol/hr). In the brain of female medaka high aromatase activity is localized in sections containing the HD, HV, Hc, NDIL, TA, and TP (85–80 fmol/hr) and highly variable levels in the POA and SC (23–70 fmol/hr). The concentration and time dependency of the exposure of male medaka to estradiol on the total brain aromatase activity and morphologic sex characteristics were determined next. Estradiol increased the activity of brain aromatase in a concentration-dependent manner at 2.5 and 25 µg/L, but the increase was lower at higher concentrations of the hormone. The effect was time dependent, gradually increasing up to the fifth day of exposure, after which it reached a plateau. Estradiol induction of brain aromatase analyzed using Lineweaver-Burke plots of saturation assays revealed a non-first-order reaction. The results indicate that a positive feedback mechanism regulates brain aromatase and imply that the sexual dimorphic distribution of aromatase may be highly sensitive to physiologic cues and environmental perturbations in fish. *Key words*: aromatase activity, endocrine disruption, medaka, sexual dimorphism. *Environ Health Perspect* 109:257–264 (2001). [Online 1 March 2001]
http://ehpnet1.niehs.nih.gov/docs/2001/109p257-264melo/abstract.html

Several classes of environmental contaminants interact with endocrine targets and may disrupt sex determination and differentiation and impair reproductive success (*1*). The consequences of endocrine disruption include reduced fertility in mammals, abnormal sexual behavior, deformities and lowered survival of hatchling birds (*2*), alteration of population sex ratios in reptiles with temperature-dependent sex determination (*3*), and feminization/masculinization in fish, both in the adult (*4*) or during sexual differentiation (*5*).

Levels of circulating hormones constitute important regulatory signals during development and in biological functions. These signals involve mechanisms of feedback loops between the central nervous system and effector organs—namely, the hypothalamus-pituitary-gonad loop of the reproductive function. The synthesis of estrogens in the brain (neuroestrogens) plays a critical role in the differentiation of sex during development (*6,7*) and in sex-specific reproductive behavior (*8*), mediating both organizational and activational effects of the circulating hormones.

In this study, we investigated sexual dimorphism in the distribution of the activity of aromatase (estrogen synthase) in the brain of the teleost medaka and its regulation by sex steroids.

Cytochrome P450 aromatase (aromatase) catalyzes the committed step of the synthesis of endogenous estrogens from androgens. In a cascade of reactions, aromatase uses the reductive equivalents from nicotinamide adenine dinucleotide (NADPH) to convert androgen (C19) to estrogen (C18) by removal of the methyl group (C19) and aromatization of the steroid A ring (*9*). Aromatase is the product of the gene family *CYP19*. Studies in mammals, including humans (*9*), birds (*10*), and medaka (*11*), support the existence of a single locus of the gene, although the presence of multiple alleles has been reported in pigs (*12*) and goldfish (*13*). The regulatory complexity of aromatase expression is achieved by use of alternative gene promoters that are spliced in a tissue-specific manner and hormonally regulated (*14,15*). Aromatase is evolutionarily conserved among the classes of vertebrates studied (*16*), and it is found in the brain, gonads, and other peripheral tissues including the placenta and the adipose tissue in mammals (*17*). In teleosts, levels of aromatase in the brain are 100- to 1,000-fold higher than in the brain of mammals (*18*). The adaptive significance of the higher levels of brain aromatase in the evolution of teleosts is not understood. Teleosts are the group of vertebrates with the widest heterogeneity and plasticity of sexual determination and differentiation. Sex reversal of the adult bluehead wrasse (*Thalassoma bifasciatum*) in response to social cues (*19*) and the different male morphs of the plainfin midshipman (*Porichthys notatus*) (*20*) illustrate this diversity.

The role of brain aromatase has been widely investigated as a regulator of sex-specific behavior in mammals (*6,21*) and birds (*22*) and of reproduction in fish (*23*). The brain is sexually dimorphic (*24*). However, the regulation and significance of brain aromatase activity are poorly understood. The high activity levels of aromatase in the brain of teleosts suggest an important role in regulatory pathways and constitute a potential target for disruption of the endocrine system by xenobiotics. The characterization of the activity of brain aromatase and its regulation are necessary to understand the mechanisms of control of reproductive function and to provide a tool for assessing endocrine disruption. In previous studies, we reported that the levels of aromatase activity in the brain of medaka were significantly higher than those in the gonads and that activity was specifically localized to areas of the brain that contained the hypothalamus (*25*).

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We thank J.S. Edmunds for providing helpful guidance during the initial course of this work.

This work was conducted in partial fulfillment of PhD requirements in Molecular and Cellular Biolology and Pathobiology from the Medical University of South Carolina for A.C.M., who was supported in part by a PhD fellowship from Fundação para a Ciência e Tecnologia, Portugal (ref. BD/ 2842/ 93-RN). This work was funded by the National and Atmospheric Administration (NOAA–NOS).

Received 17 July 2000; accepted 13 October 2000.

In the present study, we characterized the sexual dimorphism of brain aromatase in medaka and its regulation by sex steroids. Our goal was to investigate sexual dimorphism in the activity of brain aromatase and to characterize the effect of exogenous sex steroids on that activity and on the reversal of sex phenotype. The organism used was the drR strain of the medaka (*Oryzias latipes*). Medaka are widely used in environmental toxicological assays and were the first teleost in which sex reversal was obtained by exposure to sex steroids (*26,27*). We provide evidence that exposure to exogenous estradiol leads to a feminization of the localization of aromatase activity of male medaka. That modification included an increased activity in specific areas of the brain that are usually higher in the female than in the male. The effect of estradiol on the brain aromatase activity is both concentration- and time-dependent. Exposure to the aromatizable androgen methyltestosterone inhibited the activity of the enzyme to levels not measurable by our methods. This decrease occurred in all the areas of the brain that had been shown to have aromatase activity.

Materials and Methods

Test organism. Cultures of the drR strain of Japanese medaka (*O. latipes*) were maintained in our laboratory in a balanced salt solution (*27*) at 25 °C and a 16 hr light:8 hr dark cycle. Under these conditions animals breed throughout the year, starting as young as 2.5 months of age. Sexually mature individuals (> 5 months) with a body length 25–30 mm were used in our experiments.

Localization of brain aromatase activity. We used serial sections in the transverse (coronal) plane of the brain to localize areas with higher levels of aromatase activity. To identify sexual dimorphism, we obtained coronal sections from three individuals of each nontreated male and female fish. To study the effects of sex steroids, we obtained sections from males treated with 250 µg/L estradiol or 250 µg/L methyltestosterone. Males treated with DMSO (vehicle) were used as experimental controls. The experiment was performed with three animals per treatment. Individuals were quickly anesthetized in ice water and decapitated. The whole head was immediately frozen (–10°C) and mounted in a cryostat. Coronal sections of 200 µm, obtained in sequence from the most anterior area of the brain to the most posterior, were homogenized in 100 µL of phosphate buffer (in mM: 10.0 K_2PO_4, 100.0 KCl, 1.0 EDTA, 1.0 dithiothreitol at pH 7.4) and stored at –80°C. We measured aromatase activity in each section, using the tritiated water aromatase assay method described below. Levels of aromatase activity in coronal sections from DMSO-treated male reproduced those obtained from nontreated medaka [two-factor analysis of variance (ANOVA), $F_{0.05(1),1,68} = 2.15$, $p = 0.14$, $F_{0.05(1),16,68} = 38.8$, $p = 7 \times 10^{-12}$, $F_{0.05(1),16,68} = 1.63$, $p = 0.08$, respectively]. A medaka brain atlas (*28*) was used to identify which hypothalamic nuclei were present in a given coronal section by correspondence with morphologic features using a dissecting microscope.

Aromatase assay. We quantified aromatase activity using an *in vitro* assay, following the tritiated water method adapted from Thompson and Siiteri (*29*) as described by Melo et al. (*25*). Briefly, the stereospecific release of tritiated water during the conversion of the tritiated substrate ^{3}H-androst-4-ene-3,17-dione (^{3}H-A) to estrone is used as an indicator of aromatase activity. Brain homogenates were incubated for 3 hr at 25°C with 3.0 nM ^{3}H-A in the presence of 1.0 mM NADPH in a phosphate buffer (described above), in 200 µL total volume of reaction. The reaction was terminated by immersion in ice water and addition of 100 µL of 30% trichloroacetic acid (TCA). The solution was then incubated in a mix of 5% active charcoal (Norit A; Sigma, St. Louis, MO) with 0.5% dextrose (Fisher Scientific, Pittsburgh, PA) in double-distilled water, for steroid stripping for 30 min at 4°C. The mixture was then centrifuged twice at 4°C and supernatants collected. Levels of tritiated water produced were determined using 200 µL of the final supernatant by liquid scintillation counting (1211 RackBeta, Gaithersburg, MD).

To localize the activity of aromatase in the brain, we used the tritiated water assay to measure the enzyme activity separately in the homogenate of each serial section of the coronal plane of the brain. To determine the concentration- and time-dependency of the effect of sex steroids on the activity of the enzyme, we used whole-brain homogenates from individuals exposed in the *in vivo* bioassay as a source of enzyme in the tritiated water method. We investigated the kinetics of brain aromatase by performing saturation aromatase assays using different concentrations of ^{3}H-A (0.25, 0.35, 0.5, 1.5, 2.5, 4.0, and 5.5 nM). Whole-brain homogenates of male medaka exposed *in vivo* to 2.5 and 25 µg/L estradiol were used and aromatase activity was compared to control animals. We analyzed the results graphically using the double-reciprocal plot (Lineweaver-Burke plot).

In vivo *bioassay*. We evaluated the effect of sex steroids on the activity of aromatase by exposing 5–7-month-old male medaka to an estrogen and an androgen. The estrogen 1,3,5 [10]-estratriene-3,17β-diol (E_2) (Sigma), commonly designated estradiol, and the androgen 17α-methyl-4-androsten-17β-ol-3-one (MT) (Sigma), designated methyltestosterone, were dissolved in stock solutions in DMSO (Sigma). Stock solution (100 µL) was diluted in 200 mL of aquarium water to obtain the required concentrations of sex steroids in the test vessels, each holding one animal. Control animals were exposed to the equivalent volume of the vehicle (DMSO) obtaining a dilution factor of 1:1,000. Exposure lasted for 10 days (except in the time-dependency experiment, described below), with water changed every 24 hr. Animals were fed twice a day, alternating brine shrimp or flake food (VividColor, Hayward, CA). At the end of the bioassay, animals were quickly anesthetized in ice water and decapitated. For aromatase activity localization, animals (three replicates) were exposed to 250 µg/L of estradiol or methyltestosterone. At the end of the assay, the head was frozen and coronal sections were obtained as described. For the concentration- and time-dependent evaluation, we dissected whole brains with the aid of a dissection microscope and homogenized them in 300 µL of phosphate buffer; five animals per treatment were used. We evaluated the concentration dependency of the effect of estradiol on brain aromatase activity over three log concentrations of 2.5, 25, 75, and 250 µg/L of the sex steroid, and five animals per treatment were used. We investigated the time dependency using 20 µg/L of estradiol and five animals per time point. Animals were killed after 1, 3, 5, and 10 days of exposure. Control individuals were killed at the longest (10 days) sampling time. The exposure concentrations used in this study were pharmacological concentrations 1,000 times greater than those found in surface waters. To identify changes in the secondary sexual characteristics, we photographed each animal at both times, just before the start of the exposure assay and after decapitation at the end of the assay (see description below).

Effects of sex steroids on sex phenotype. To identify the effects of estrogens and androgens on the sex phenotype, we determined localization of brain aromatase activity in coronal sections as described above. We then compared the aromatase activity to alterations of secondary sexual characteristics. These characteristics included the genital papilla, the presence of papillary processes on the anal fin, and the notch on the dorsal fin (*26,27*). The urogenital papilla is enlarged in females and small in males. Both the presence of papillary processes at the posterior region of the anal fin and a notch in the dorsal fin between the last and second last fin rays occur only in males. Enlargement of the urogenital papilla and a decrease in the notch size and in the

number of papillary processes in males was considered reversal of the secondary sexual characteristics in medaka. These characteristics were photographed (FlashPoint FPG 3.10; FlashPoint, North Reading, MA) before and after the *in vivo* bioassay using a dissection microscope (Leica MZ12, magnification 30×; Leica, Deerfield, IL).

Statistical analysis. Analysis of the experiments using coronal sections of the brain required a factorial ANOVA to assess simultaneously the localization of aromatase activity in the sequence of brain sections and the effect of sex or of estradiol treatment. We used a two-way ANOVA with equal replication in which the sex or the estradiol treatment (in the respective experiments) constituted one factor and the localization of aromatase activity to the coronal brain sections obtained in sequence constituted the second factor of the analysis. Considering the increased probability of obtaining a statistical type I error due to the large difference of number of levels in each of the factors (2 in the sex and estradiol treatment and 17 in the factor of the sections sequence), a two-way ANOVA using only a subsample of the sections was also performed. The subsample of sections selected corresponded to an area of the brain of interest in this study. The results of the analysis performed with the complete set of data and with the subsample were equivalent. To analyze the data of brain sections from the experiment of exposure of males to sex steroids, we used the two-way ANOVA as follows: First, all the data of sections from control, estradiol, and methyltestosterone treated animals were used. Then we performed the analysis to compare the control to the estradiol-treated animals. Finally, as we did to compare male-to-female brain aromatase activity, we selected a subset of sections from the control and estradiol-treated animals and performed the same analysis. We analyzed the data from the concentration- and time-dependent experiments using the one-way ANOVA. The groups contributing to the significance of the analysis were identified by the application of the multiple-comparison Tukey-Kramer test. To compare the results from the enzyme saturation assays, we linearized the data in Lineweaver-Burke double-reciprocal plots, and applied the linear analysis of regression. The effect of estradiol on the morphology of secondary sex characteristics was defined categorically as an all-or-none type of effect. The results are presented as the percentage of animals in each treatment that showed the phenotypic change.

Results

We examined sexual dimorphism of brain aromatase by measuring aromatase activity in serial coronal frozen sections in male and female fish. The two-factorial ANOVA showed that the activity of aromatase is significantly different between males and females, and it also is different between sections of different areas of the brain, showing an interaction between the effect of these two factors ($F_{0.05(1),1,68} = 10.91$, $p = 0.001$; $F_{0.05(1),16,68} = 10.4$, $p = 1.17 \times 10^{-12}$; and $F_{0.05(1),16,68} = 4.15$, $p = 1.74 \times 10^{-5}$, respectively). Considering the inequality of the number of levels between the two factors [17 levels (sections) for the localization factor and 2 levels (male and female) for the other factor], we used a subsample of brain sections (sections 4, 5, 9, 10, and 11), corresponding to areas of the brain that contain hypothalamic nuclei of interest in this study, in a two-factor ANOVA.

The results of this analysis showed that the activity of brain aromatase in these sections is significantly different between males and females and detected a contribution of the localization to that difference ($F_{0.05(1),1,20} = 10.52$, $p = 0.004$; $F_{0.05(1),4,20} = 2.37$, $p = 0.08$; and $F_{0.05(1),4,20} = 11.26$, $p = 5.98 \times 10^{-5}$, respectively). Brain aromatase was highest in sections 4–6 of male and in sections 6–11 of female. One-way ANOVA performed on individual sections showed that the activity of brain aromatase in section 4 was significantly higher in the male (mean ± SE, 63 ± 9.4 fmol/hr) than in the female (24 ± 4.4 fmol/hr; $F_{0.05(1),1,4} = 14.09$, $p = 0.019$; Figure 1). In sections 9 and 10 brain aromatase activity was significantly higher in the female (80 ± 10.5 and 54.6 ± 16.3 fmol/hr, respectively) than in the male (6.1 ± 1.2 and 2.8 ± 0.3 fmol/hr; $F_{0.05(1),1,4} = 48.34$, $p = 0.002$; $F_{0.05(1),1,4} = 48.3$, $p = 0.002$, respectively for sections 9 and 10). These sections correspond to an area of the brain that contains specific hypothalamic nuclei: the preoptic nuclei (POA) and suprachiasmatic nucleus (SC) in sections 4 through 7, and the nuclei periventricularis dorsalis (HD), ventralis (HV), caudalis (Hc), nucleus lateralis (LH), tuberis anteriores (TA), and tuberis posteriores (TP) in sections 8 through 11 (Figure 2).

These results reveal sexual dimorphism in the localization of brain aromatase activity and suggest the involvement of specific hypothalamic nuclei, which are contained in those sections, in the regulation of male or female functions. When male medaka were exposed to 250 µg/L estradiol, the localization of higher levels of aromatase activity was statistically different. The application of the two-factorial ANOVA showed that at the level of confidence of 5% ($\alpha = 0.05$), the difference between the treatments did not depend on the localization of the activity in the brain sections ($F_{0.05(1),1,68} = 41.9$, $p = 1.24 \times 10^{-8}$; $F_{0.05(1),16,68} = 18.15$, $p = 1.55 \times 10^{-18}$; $F_{0.05(1),16,68} = 1.53$, $p = 0.113$, respectively, for estradiol treatment, sections, and interaction). To address the possible occurrence of a type I error due to the inequality of levels in each factor (2 for hormone

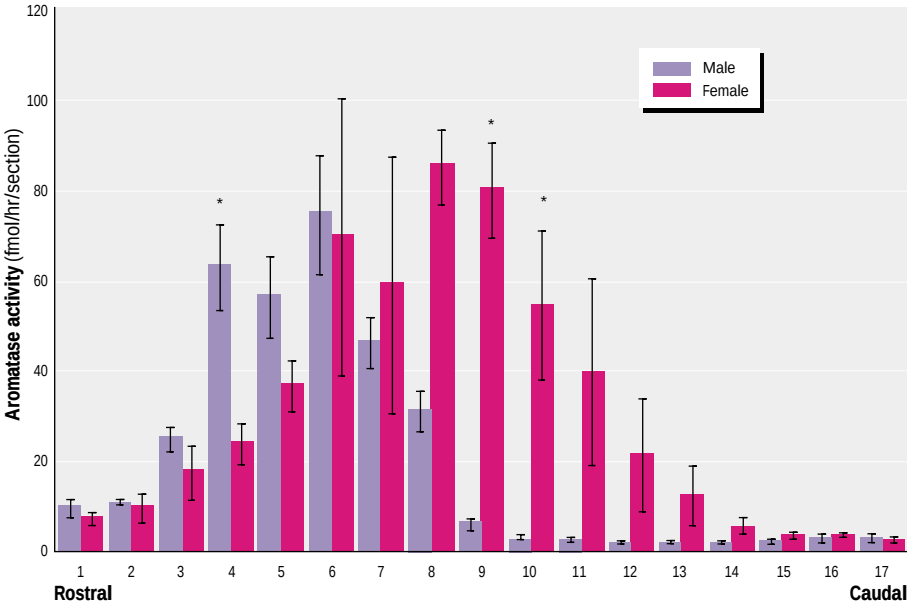

Figure 1. Aromatase activity in coronal sections of the brain of male and female medaka. Aromatase activity is described in femtomoles of tritiated estradione (^{3}H-A) converted to estrone per hour of incubation, as indicated by the quantification of the tritiated water released in the reaction. Homogenates of brain coronal 200 µm sections were incubated in a phosphate buffer with tritiated androstenedione and in the presence of NADPH, for 3 hr at 25°C. Each bar represents the mean ± SE of each section from three individuals. Asterisks represent a significant difference between male and female in the corresponding brain section. Sections are in sequence (1–17), from the rostral to the caudal side of the brain.

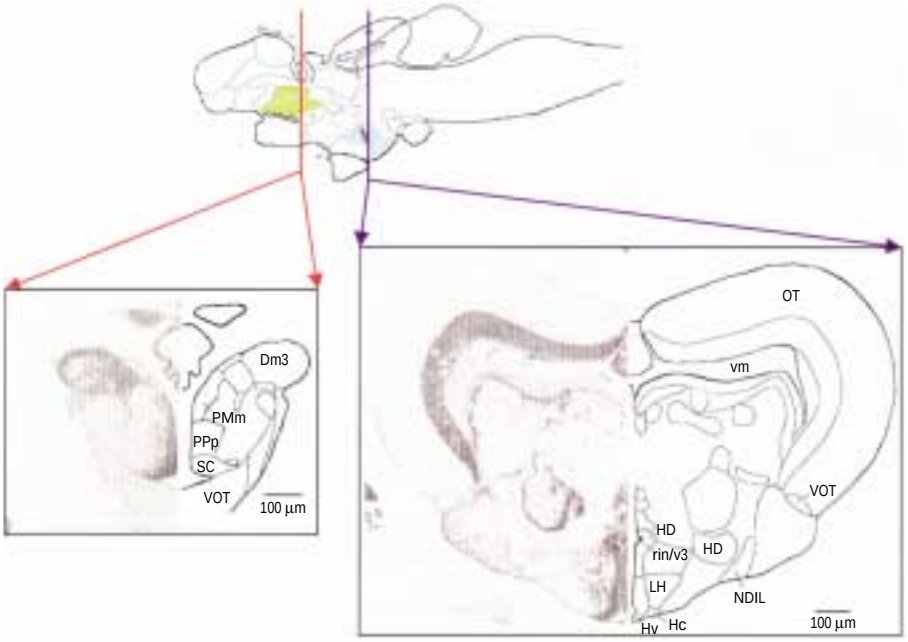

Figure 2. Schematic representation of the brain of medaka, adapted from Anken and Bourrat (*28*) and modified using Photoshop 5.0 (Adobe, Mountain View, CA). Abbreviations: Dm3, area medialis of dorsalis telencephali; Hc, hypothalamus periventricularis caudalis; HD, hypothalamus dorsalis; HV, hypothalamus ventralis; LH, nucleus lateralis hypothalamicus; NDIL, nucleus diffusus of lobus inferiores of hypothalamus; OT, optic tectus; PMm, nucleus preopticus magnocellularis pars magnocellularis; PPp, nucleus preopticus parvocellularis posterioris; rin/v3, recessus infundibularis of ventriculus tertius; SC, suprachiasmatic nucleus; vm, ventriculus mesencephali; VOT, tractus opticus ventralis. An outline of a midsagittal section of the brain of medaka is shown at the top. Highlighted in yellow and blue are hypothalamic nucleus, and the overlying lines approximately localize sections 6 (red line) and 9 (blue line) where higher levels of aromatase activity were found, respectively, in the male and in the female. Connected by arrows are the coronal sections corresponding to the area localized by the lines overlying the mid-sagittal outline. For clarity, the right half of the coronal sections outline the structures identified in that area.

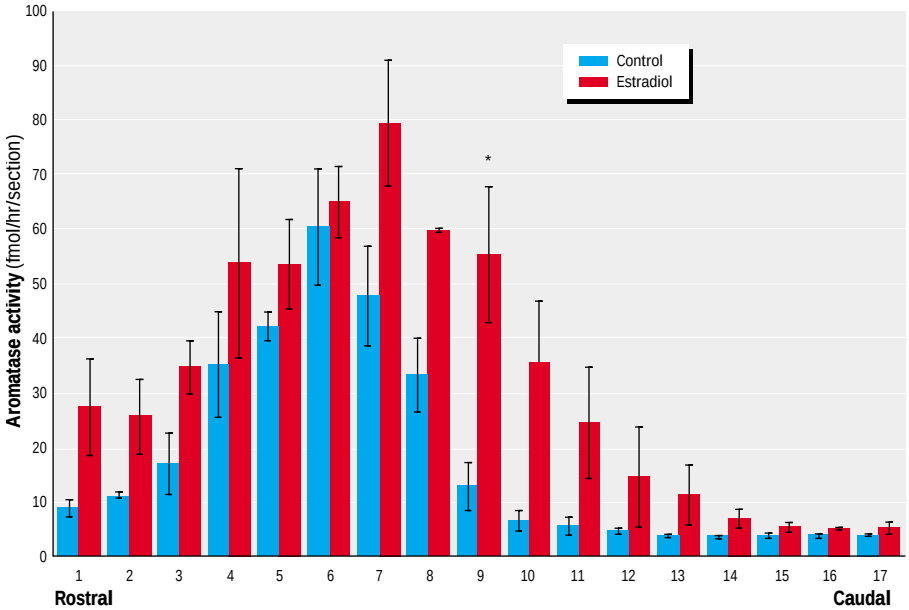

Figure 3. Effect of estradiol on the brain aromatase activity phenotype of male medaka showing aromatase activity in coronal brain sections of estradiol-treated male fish compared to that in control fish (treated with the vehicle DMSO). Aromatase activity is expressed in femtomoles of converted tritiated estradione to estrone, as indicated by the measurement of tritiated water released in the reaction. Individuals were treated with 250 µg/L estradiol for 10 days, sacrificed, and coronal 200-µm sections of the brain obtained and homogenized. Brain section homogenates were incubated in a phosphate buffer, in the presence of 1 mM NADPH, for 3 hr at 25°C. Sections 1–17 are in sequence from the rostral to the caudal side of the brain. Bars are the mean ± SE of sections from three individuals. Asterisks represent significant difference (ANOVA, $n = 6$, $p < 0.05$) between the two experimental groups, in the indicated brain section.

treatment and 17 for brain sections), we applied another two-factorial ANOVA to a subsample of sections (sections 3, 4, 5, 9, and 10) that correspond to regions of the brain of interest in this study. The results of analysis of the subsample agreed with those obtained using the entire set of data ($F_{0.05(1),1,20} = 16.45$, $p = 0.0006$, $F_{0.05(1),4,20} = 4.72$, $p = 0.0076$; $F_{0.05(1),4,20} = 0.82$, $p = 0.5$, respectively, for hormone treatment, sections, and interaction). The activity of aromatase was increased in sections 8–11 relative to the control (Figure 3) with a statistically significant increase in section 9 (12.7 ± 4.3, and 55.0 ± 12.3 fmol/hr in control and estradiol treated fish, respectively, $F_{0.05(1),1,4} = 10.4$, $p = 0.03$) suggesting a feminization of the brain due to the higher levels of estradiol. Exposure to methyltestosterone inhibited the activity of aromatase in all the brain sections of male medaka to levels near the lower limit of sensitivity of our assay (Figure 4) ($F_{0.05(1),2,102} = 85.29$, $p = 1.69 \times 10^{-22}$; $F_{0.05(1),16,102} = 19.9$, $p = 1.85 \times 10^{-24}$; $F_{0.05(1),32,102} = 4.2$, $p = 1.6 \times 10^{-8}$, respectively for hormone treatment, sections, and interaction).

To determine the concentration-dependent effects of estradiol on the activity of brain aromatase and on the reversal of the secondary sex characteristics, we used log dilutions of 250 µg/L of estradiol (E2) to perform *in vivo* assays with male adult medaka. Brain aromatase activity was significantly increased (ANOVA, $F_{0.05(1),4,23} = 10.65$, $p = 0.0001$; Tukey-Kramer, $q_{0.05,20,5} = 2.297$, $p < 0.05$) following treatments with 2.5 and 25 µg/L E2 when compared to controls (Figure 5). However, the higher concentrations tested (75 and 250 µg/L), were not statistically different from those of controls. In parallel with the study of the effect of estradiol on brain aromatase, the reversal of the morphologic secondary sex characteristics of the treated animals was next investigated with the aid of a dissection microscope. Of three separate sex-dependent markers—the genital papilla, the papillary processes, and the dorsal fin notch—only the genital papillae exhibited the female phenotype. The reversal of the sex phenotype of the genital papilla to female was observed in 100% of the males in all the concentrations of estradiol that were tested and 0% in the control animals (Figure 5).

The time dependency of the effect of estradiol on the activity of brain aromatase and on the reversal of the secondary sex characteristics was investigated next. *In vivo* bioassays were performed using 20 µg/L of estradiol. Time points were 1, 3, 5, and 10 days. The activity of brain aromatase in fish exposed to 20 µg/L estradiol increased progressively with time up to day 5, after which a plateau was observed (Figure 6). The

increase in aromatase activity was not significantly different from that of controls at day 1, but it was significantly different at all other time points (3, 5, and 10 days), with aromatase activity significantly higher on day 5 relative to day 3 (ANOVA, $F_{0.05(1),4,24} = 19.44$, $p = 1.15 \times 10^{-6}$; Tukey-Kramer, $q_{0.05,20,5} = 2.297$, $p < 0.05$). Of the three sexually dimorphic characteristics observed, only the genital papilla was affected by estradiol in a time-dependent manner in our assays. There was no change in morphologic sex phenotype after 1 day of exposure to each of the test concentrations. In the fish exposed for 3 days, there was a reversal to female phenotype in 40% of the animals in the assay with 20 µg/L estradiol. At days 5 and 10 there was 100% reversal to female phenotype of the genital papillae (Figure 6). The papillary processes were affected transiently (20% change on day 3, but no change in samples of day 5 and day 10). There was no observable effect of estradiol on the dorsal fin notch in these experiments. In the experiments testing a range of concentrations of estradiol, the genital papilla was affected in 100% of the animals at every concentration tested.

To investigate the enzyme kinetics of brain aromatase in estrogen-treated medaka, we performed Lineweaver-Burke analysis of enzyme saturation assays. Saturation curves obtained with brain homogenates of control fish and fish exposed to 2.5 and 25 µg/L estradiol in an *in vivo* assay were compared. First-order Michaelis-Menten (K_m) type of curve was observed with the homogenates of control fish, with an apparent K_m of 1.0 nM (Figure 7). The saturation curves obtained with the brain homogenates from estradiol-treated fish failed to reach a plateau. An apparent K_m of about 5nM, different from that obtained with the control fish, was extrapolated. This indicates that the enzyme is not following a first-order type of kinetics. The effect of estradiol implies the activity of mechanisms other than a simple increase of enzyme concentration.

Discussion

We identified sexual dimorphism in brain aromatase activity of medaka. The male phenotype of brain aromatase activity is characterized by the presence of high levels of activity confined to the coronal sections of the brain that contain the POA and the SC. Low levels of activity existed in all the other brain areas, including those containing the HD, HV, and Hc. The female phenotype is characterized by the presence of high aromatase activity in coronal sections of the brain that contain the periventricular nuclei HD, HV, and Hc, the NDIL, and the TA and TP. Aromatase activity in sections 6 and 7 of the female is highly variable (range 23 to 70 fmol/hr).

The organizational structure of the hypothalamus of teleosts comprises functional nuclei as discrete structures distributed along the longitudinal axis of the brain, in a medial–ventral localization. An experimental advantage of this organization is the ability to analyze groups of nuclei by sectioning the brain along the longitudinal axis. Using this

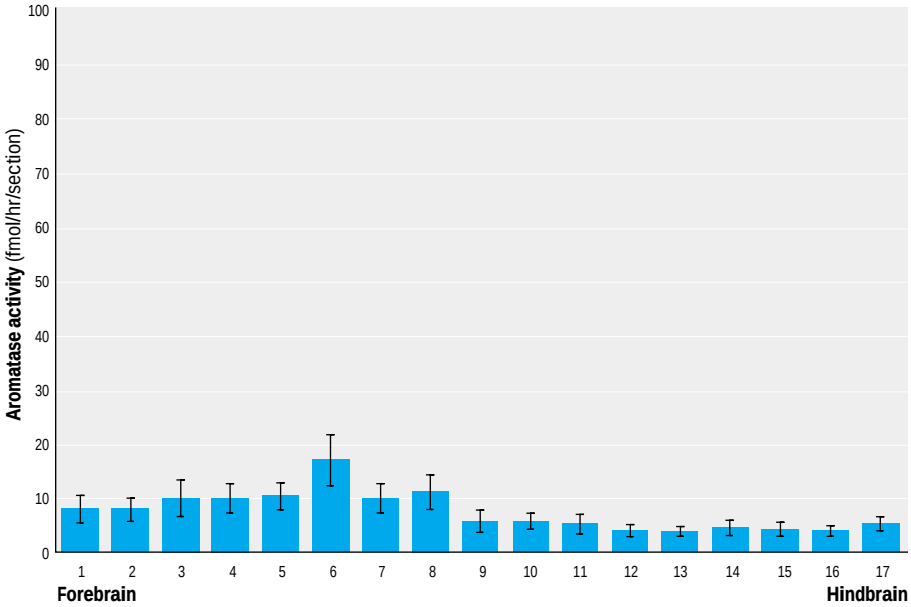

Figure 4. Effect of methyltestosterone on the brain aromatase activity of male medaka. Aromatase activity is expressed in femtomoles of converted tritiated estradione to estrone, as indicated by the measurement of tritiated water released in the reaction. Sexually mature male medaka were treated with 250 µg/L methyltestosterone for 10 days and then were killed. Brains were immediately sectioned, homogenized, and stored at –80°C until use in aromatase assay. Sections 1–17 are in sequence from the fore- to the hindbrain. Each bar is the mean ± SE of each section from three individuals.

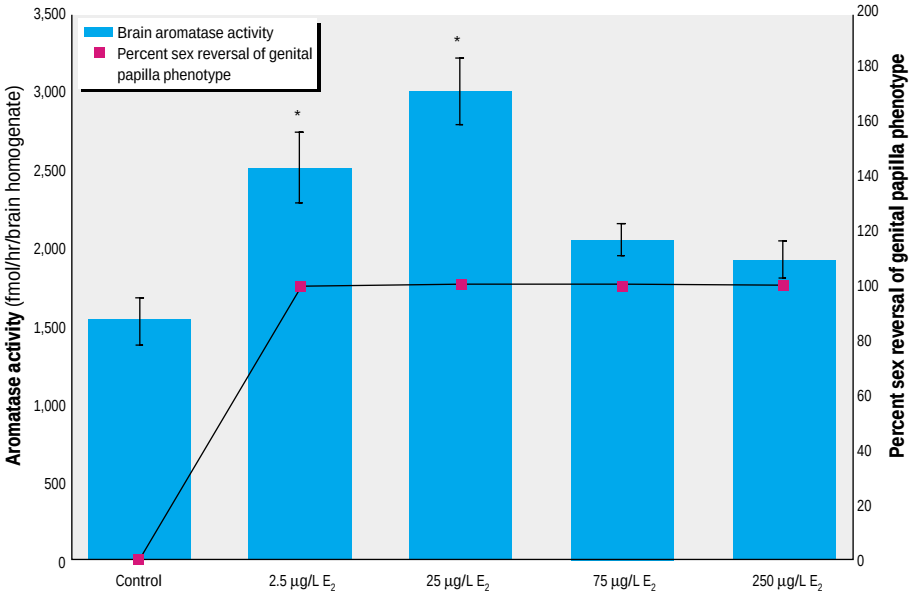

Figure 5. Concentration-dependent effect of estradiol on the activity of brain aromatase and effect on the sexual phenotype of the genital papillae. The results corresponding to these two independent variables—brain aromatase activity and morphologic changes in the secondary sexually dimorphic characteristics—were obtained in the same experiment and from the same animals. Aromatase activity is expressed in femtomoles of converted tritiated estradione to estrone, as indicated by the measurement of tritiated water released in the reaction. Male medaka were treated for 10 days with 2.5, 25, 75, or 250 µg/L of estradiol and killed, and homogenates of the dissected whole brain were obtained. Each bar is the mean ± SE of the activity of brain aromatase of five individuals of each treament. The genital papillae were photographed in a dissecting microscope (magnified 30×) before and after treatment with estradiol. Control animals were treated with DMSO, in volume equivalent to that used to dilute the steroid (< 1/1,000).
*Tukey-Kramer $q_{0.05,20,5} = 2.297$, $p < 0.05$.

approach and making the correspondence between the brain sections obtained in sequence from the foremost region of the brain to those of an atlas of the medaka brain (*28*), we identified those hypothalamic nuclei contained in the sections where aromatase activity in males differed from that in females. In our previous work, a three-dimensional representation of brain aromatase activity localized the highest activity levels to a medial ventral area of the brain, the area that contains the hypothalamic structures (*25*). In the work presented here, we found that in the male, high aromatase activity occurs in a region of the brain that contains the preoptic hypothalamic nuclei and the suprachiasmatic nucleus (sections 4–7, with the highest levels of activity in section 6). All the remaining sections of the male brain (1–3 and 9–17) showed an activity of aromatase < 25 fmol/hr. In the longitudinal profile of aromatase of the female brain, higher levels of activity were found in sections 6–11. Only sections 1–4 and 12–17 (compare to 1–3 and 9–17 in the male) have aromatase activity < 25 fmol/hr. High variability among female individuals was found in sections 6 and 7, but sections 8, 9, 10, and 11 consistently had significantly higher levels than in the males. Sections 8–11 contain the HD, HV, Hc, LH, TA, TP, and NDIL. Our results demonstrate sexual dimorphism of the activity of aromatase in the sections that contain these nuclei. They are localized to an area of the brain dorsal to the pituitary, which appears to correspond to the median eminence in mammals, an area important in the release of regulatory factors to the pituitary. The sexual dimorphism of aromatase activity in this area of the brain of medaka is probably involved in the sex-specific regulation of the gonadal–pituitary–hypothalamic feedback loop. Of consideration is the low aromatase activity in the gonads of medaka (*25*) compared to that in the brain. This suggests that while the regulatory levels of circulating estrogens in mammals depend mostly on the ovarian synthesis of estrogens in response to neuroendocrine cues, in teleosts those levels depend strongly on local synthesis of estrogens in specific areas of the brain.

The high variability between individual females in the dorsal preoptic area might be related to a cyclic regulatory control of ovulation or to different ovulatory stages. In mammals (*30*) and quail (*31*), a sexually dimorphic nucleus (SDN) has been localized to the corresponding preoptic area. The presence of aromatase in these nuclei suggests that the local synthesis of estrogens plays a role in controlling the release of gonadotropin-releasing/inhibiting factors by the parvocellular neurons. Another possible source of local variability of aromatase activity may be the anterior pituitary. Aromatase activity in the anterior pituitary was reported in a teleost species, the longhorn sculpin (*Myoxocephalus octodecimspinosus*) (*18*). Our data suggest that the aromatase activity in the preoptic nuclei of medaka is sexually dimorphic. A study of the aromatase activity in the pituitary would reveal whether these nuclei are characterized by consistently lower levels of aromatase activity in females relative to males, similar to those observed in the rostral region of the POA, or by the observed variability. These fluctuations of aromatase activity may be related to the reproductive cycle of the females.

We demonstrated that estradiol administered to adult males via the salt solution alters the anterior–posterior profile of brain aromatase activity in the neuroaxis to resemble that of the female phenotype. Treatment of males with estradiol produced a shift of the sections with high aromatase activity levels, from those containing the POA to those more caudal areas of the brain. Specifically, estradiol increased the levels of aromatase activity in sections 8–11. Aromatase activity in these brain sections was sexually dimorphic, with low activity levels in the male and higher levels in the female. The induction of brain aromatase activity by estradiol has been shown in medaka (*25*), goldfish (*23*), rats (*32*), and quail (*33*). Sexual dimorphism in total levels of enzymatic activity has been shown in goldfish (*23*). In teleosts, the effect of estradiol to elevate aromatase activity occurs in a region of the brain corresponding to the medial basal hypothalamus. The mechanisms underlying the regulatory pathways that give origin to specific effects in distinct brain nuclei are not well understood. Direct (via the estrogen receptor) and/or indirect (neurotransmitters and other factors) mechanisms may be involved.

Brain aromatase was inhibited by an aromatizable androgen in all areas of the brain where activity was identified. Studies of the effect of androgens on brain aromatase have not led to consistent conclusions. In the ringdove (*34*), rat (*35*), quail (*31*), and goldfish (*23*), the aromatizable androgens induce brain aromatase activity. Reports have also indicated inhibition in cultures of neural tissue (*36*) in whole-brain homogenates of medaka (*25*) and showed no effect in certain prenatal stages in rat (*24*). These inconsistent results of androgen regulation of brain aromatase activity clearly demonstrate the interplay of diverging mechanisms by which androgens affect the activity of brain aromatase.

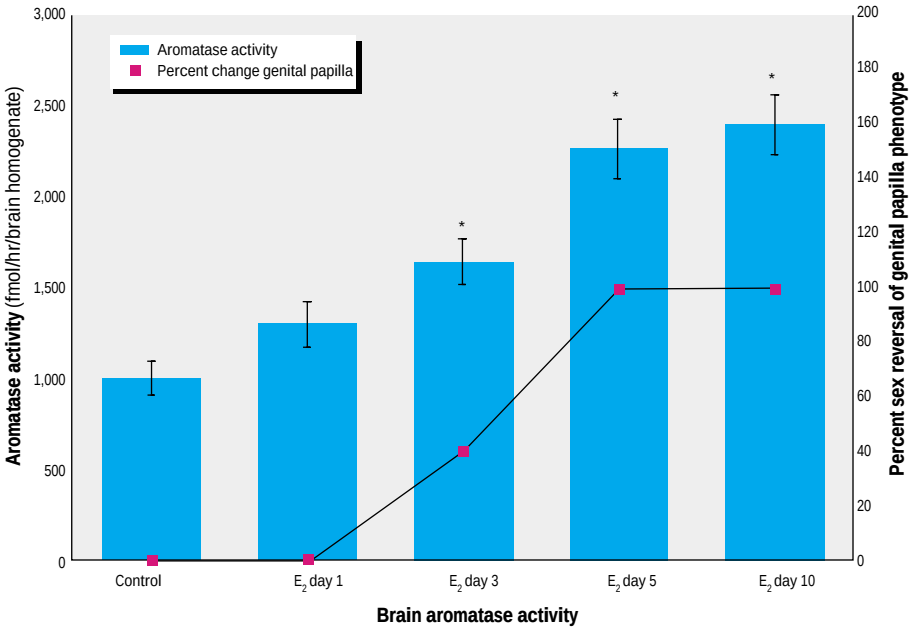

Figure 6. Time dependency of the effect of estradiol on the activity of brain aromatase and on the sexual phenotype of the genital papillae. The results corresponding to these two independent variables—brain aromatase activity and morphologic changes in the secondary sexual characteristics—were obtained in the same experiment and from the same animals. Aromatase activity is expressed in femtomoles of tritiated estradione converted to estrone, as indicated by the measurement of tritiated water released in the reaction. Male medaka were treated with 20 µg/L estradiol for 1, 3, 5, or 10 days and killed, and whole-brain homogenates were used in the aromatase assay. Each bar is the mean ± SE of brain aromatase activity of five individuals in each treatment. The genital papillae were photographed in a dissecting microscope (magnified 30×) before and after the treatment with estradiol. Control animals were treated with DMSO, in volume equivalent to that used to dilute the steroid (< 1/1000).
*Tukey-Kramer $q_{0.05,20,5} = 2.297$, $p < 0.05$.

Estrogen exposure has two actions on brain aromatase in male medaka: elevated total levels of aromatase activity and localized expression in the medial hypothalamic nuclei. The total levels of brain aromatase activity increased with 2.5 and 25 µg/L estradiol; yet at higher doses (75 and 250 µg/L), significant increases were not found. Alternative regulatory pathways of brain aromatase are probably triggered by levels of estrogens above a certain threshold, which was achieved in our experiment with the exposure to 75 µg/L of exogenous estradiol. Induction of aromatase in the medial hypothalamus was observed at 250 µg/L, a concentration where a total brain aromatase level increase was not observed. Whether this medial distribution of brain aromatase occurs at lower doses of estradiol has not yet been determined.

The sexual phenotype of the genital papilla was reversed to the female morph in males exposed to estradiol. The reversal of this phenotype was independent of the concentrations of estradiol used in our experiments but was dependent on the time of exposure, following the same pattern of change of brain aromatase and reaching 100% of sex phenotype reversal after 5 days of exposure. The effect of estradiol on the genital papilla seems to be a direct effect of estradiol on that tissue, leading to cellular and physiologic processes that increase the volume of that tissue, probably via estrogen receptor. The presence of estrogen receptors in tissues of accessory organs (placenta, mammary glands) and other tissues (adipose, bone) is well known in mammals but has not been investigated in teleosts.

The increase of aromatase activity by estradiol implies a positive feedback in which the enzymatic activity is upregulated by the reaction product. This positive feedback has been suggested (*37*), although the mechanisms are still not well understood. Enzyme induction is a common biological mechanism to increase total enzymatic activity. We investigated the enzyme kinetics of aromatase by performing Lineweaver-Burke plots to analyze saturation assays. We determined that the brain aromatase of medaka has an apparent K_m of 1 nM. The apparent K_m of brain aromatase of males treated with estradiol showed a different value (5 nM). We conclude that the induction of genetic expression of aromatase is not the only mechanism regulating the activity of brain aromatase. Neurotransmitters such as dopamine may also modulate the neurosynthesis of estrogens by aromatase. It has also been suggested that dopamine may interact directly with aromatase (*38*). The catechol metabolites of estrogen can compete with dopamine for degradation by catechol-O-methyl transferase, increasing the concentration of dopamine. Cell volume increase and the increase of the number of cells can also increase the activity of aromatase in the brain.

In summary, we identified sexual dimorphism of brain aromatase activity in medaka. Estradiol alters the activity of brain aromatase in adult male medaka by inducing an anterior–posterior profile of aromatase activity distribution in the medial hypothalamus characteristic of the female. A positive regulation of estradiol on the rate-limiting enzyme for its synthesis comprises a positive feedback loop. This positive feedback of estradiol on the activity of aromatase is a mechanism that can lead to very high levels of estrogens in response to a small stimulus and is a potential target that will lead to endocrine disruption in the presence of small alterations of estrogen levels.

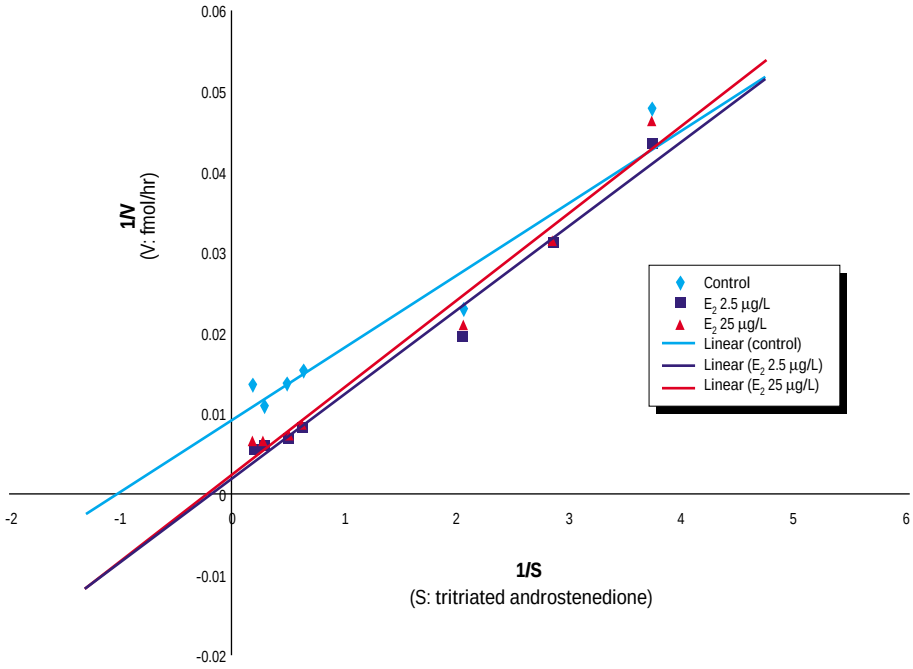

Figure 7. Lineweaver-Burke plot of brain aromatase activity of male medaka exposed *in vivo* to 2.5 or 25 µg/L estradiol and of nontreated animals. Aromatase activity saturation assays were performed using whole-brain homogenates, incubated with increasing concentrations of ^{3}H-A (0.25, 0.35, 0.5, 1.5, 2.5, 4.0, and 5.5 nM). Values are means of triplicates from a representative aromatase saturation assay performed using the brain homogenate from one individual from each treatment. Aromatase activity is expressed in femtomoles of converted tritiated estradione to estrone. The assay was repeated three times with equivalent results. Control: $y = 0.0089x + 0.0092$; $R^2 = 0.9338$. E_2 2.5 µg/L: $y = 0.0104x + 0.0021$; $R^2 = 0.9801$. E_2 25 µg/L: $y = 0.0107x + 0.0025$; $R^2 = 0.9739$.

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