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Low-Dose Bisphenol A Induces Brain Aromatase (*cyp*19*a*1*b*) Expression in Male Zebrafish (*Danio rerio*): Implications for Endocrine-Active Substance Screening

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Abstract

Environmental endocrine-disrupting chemicals (EDCs) are linked to adverse reproductive and neuroendocrine outcomes in vertebrates. Bisphenol A (BPA), a high-production monomer used in plastics and resins, exhibits estrogenic activity and is detected in aquatic environments. In teleosts, the brain aromatase isoform cyp19a1b is an estrogen-responsive transcript enriched in radial glia and neurons, suggesting utility as a central nervous system biomarker of xenoestrogen exposure. Here, we partially cloned zebrafish cyp19a1b cDNA, examined its tissue distribution and neuroanatomical localization, and tested whether short-term exposure to 17β-estradiol (E2) or BPA modulates cyp19a1b expression in adult male brain. Semi-quantitative RT-PCR showed that cyp19a1b was abundant in female brain but scarce in peripheral tissues; in males, basal brain expression was low and testis was negative. *In situ* hybridization using a clone-derived, DIG-labeled probe revealed widespread labeling across the forebrain, optic tectum, cerebellar Purkinje cell layer, hypothalamic periventricular neurons and pituitary. Five-day BPA exposure elicited a non-monotonic (U-shaped) response, with significant induction at both low (10^{-8} M) and high (10^{-5} M) concentrations. These results indicate that adult male zebrafish brain retains inducible aromatase capacity and that cyp19a1b is sensitive to environmentally relevant low-dose BPA. Collectively, our findings support brain cyp19a1b as a practical neuroendocrine biomarker for endocrine-active substance screening and environmental monitoring and demonstrate that semi-quantitative RT-PCR can detect low-dose, non-monotonic dose-response behavior.

Keywords

Bisphenol A, cyp19a1b, Endocrine Disruptor, Zebrafish

1. Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous agents that mimic, antagonize, or otherwise perturb endocrine signaling. Decades of research have associated EDC exposures with altered sex differentiation, aberrant secondary sexual characteristics and reduced fertility across wildlife and humans [1]-[6]. Bisphenol A (BPA) is emblematic among EDCs: it can leach from food-contact plastics and resins, is detectable in aquatic habitats and organisms, and exhibits estrogenic and anti-androgenic activities in vitro; developmental exposures disturb embryogenesis and gonadal differentiation in multiple vertebrate models [7]-[11]. Field observations of feminization-associated pathology in fish from contaminated waterways underline the need for tractable mechanistic models linking molecular readouts to organismal outcomes [12].

Teleosts possess duplicated aromatase genes, with one isoform expressed mainly in the ovary (cyp19a1a) and the other in the brain (cyp19a1b). The brain-specific isoform cyp19a1b is enriched in radial glia and neurons and is transcriptionally regulated by estrogens via estrogen-responsive elements (EREs) [13] [14]. Local neurosteroidogenesis in the brain modulates synaptic plasticity, neurogenesis and neuroendocrine output [15]-[17]; dysregulated aromatase expression could therefore re-tune neuronal circuits and neuroendocrine axes. As a CNS-localized biomarker of estrogenicity, cyp19a1b has three advantages: anatomical specificity (restricted mainly to brain and pituitary), mechanistic anchoring to estrogen receptor (ER) signaling, and operational tractability by RT-PCR and *in situ* hybridization. Moreover, it may capture non-monotonic low-dose phenomena that complicate risk extrapolation from high-dose data [18].

The zebrafish (*Danio rerio*) is a small cyprinid teleost that has become a widely used model in developmental biology, genetics and toxicology. Zebrafish offer several practical advantages for endocrine disruption research: they are easy and inexpensive to maintain, have high fecundity and external fertilization, and their embryos and larvae develop rapidly and transparently, enabling direct visualization of organogenesis and brain development. The hypothalamic-pituitary-gonadal axis and steroidogenic pathways are broadly conserved with other vertebrates, allowing mechanistic insights that are relevant beyond fish. Chemical exposures can be performed simply by adding test substances to the water, ensuring whole-body exposure at defined nominal concentrations. In addition, the genes encoding both ovarian and brain aromatase isoforms, including cyp19a1b, have been cloned and characterized in zebrafish [11] [13] [14], providing molecular tools to assess estrogenic activity at the transcriptional level. These characteristics make zebrafish particularly suitable for screening endocrine active substances and studying their

effects on neuroendocrine endpoints.

In the present study, we use zebrafish to test the hypothesis that adult male brain retains an inducible cyp19a1b response to estrogens and xenoestrogens. Specifically, we 1) partially cloned cyp19a1b and optimized semi-quantitative RT-PCR, 2) defined tissue distribution and neuroanatomical localization, and 3) examined whether short-term exposure to E2 or BPA modulates cyp19a1b expression in adult male brain—a sentinel context for detecting feminization signals in males. The design, doses and analytical workflow follow the authors' original experimental materials, and all expression analyses were performed using semi-quantitative RT-PCR. Note that this gene was historically referred to as *cyp*19*b* in some zebrafish literature, but throughout this paper we use the current nomenclature, cyp19a1b.

2. Materials and Methods

2.1. Animals, Ethics and Husbandry

Juvenile zebrafish (Danio rerio) were obtained from Ornament Fish Kansai Ltd. (Japan), fed a formulated diet (~45% protein, 5% lipid, 2% fiber) and reared to ~3 cm standard length before experiments. According to supplier information, the formulated diet was not supplemented with soy meal or other known phytoestrogen-rich ingredients; thus, unintended dietary estrogen exposure was considered minimal. Sex was determined based on external dimorphism (body size and coloration; **Figure 1(A)**). Fish were acclimated for 3 days in dechlorinated fresh water and individually housed in glass vessels during exposure (**Figure 1(B)**). All procedures were approved by the Animal Experimentation Committee of Kyoto Prefectural University (approval no. KPU200819) and conformed to institutional guidelines.

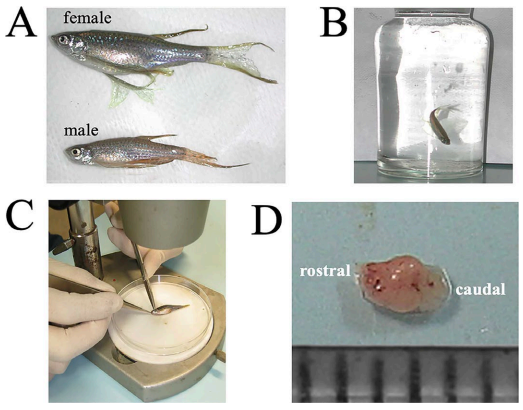

Figure 1. Gross dimorphism of adult zebrafish, exposure setup and brain sampling. (A) Representative female (top) and male (bottom) zebrafish. Males are smaller and exhibit a slightly reddish body hue relative to females; these external features were used for sexing prior to exposures. (B) Individual housing of a male zebrafish in a glass vessel containing test solutions (E2 or BPA) prepared in ethanol; control fish were maintained in fresh water. (C) Brain dissection under MS-222 anesthesia using a stereomicroscope. (D) Isolated brain (~2 - 3 mm in length) removed under a stereomicroscope and processed for semi-quantitative RT-PCR or *in situ* hybridization.

2.2. RNA Extraction, cDNA Synthesis and Partial Cloning of cyp19a1b

Total RNA from female brain was isolated with TRIzol (Gibco BRL/Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. RNA concentration and purity were assessed spectrophotometrically. One microgram RNA was reverse transcribed using oligo(dT) primers in a 20 μL reaction (First Strand cDNA Synthesis Kit; TOYOBO, Osaka, Japan).

PCR was performed in a 25 μL reaction containing 1 μL cDNA, 0.2 mM dNTPs, 0.5 μM of each primer, 1× reaction buffer and 0.5 U rTaq DNA polymerase (Promega, Madison, WI, USA). Primers for zebrafish cyp19a1b were designed from zebrafish ESTs by N.F.: forward 5'-ATTATTCGCCCTCCTGTCATTTT-3' and reverse 5'-AGCCACCTGTATACTTTCCCTCAA-3'. Cycling conditions were 94˚C for 3 min followed by 35 cycles of 94˚C 30 s, 55˚C 1 min and 72˚C 1 min, with a final extension at 72˚C for 5 min. Products were resolved on 1.5% agarose/TAE gels stained with ethidium bromide and visualized (**Figure 2(A)**); bands were excised and purified using silica-membrane spin columns (Invitrogen).

Amplicons (~500 bp) were subcloned into pCR II-TOPO (Invitrogen) and transformed into TOP10F' competent *Escherichia coli* (Invitrogen). Plasmids were prepared (Qiagen, Hilden, Germany), screened by EcoRI digest, and sequenced by N.F. (Sawady Technology Inc., Japan). Sequence analysis confirmed that the cloned fragment corresponded to zebrafish brain aromatase cyp19a1b (**Figure 2(B)**).

To determine appropriate cycle number for semi-quantitative RT-PCR, we amplified cyp19a1b from female brain cDNA using 23 - 39 cycles and quantified band intensity by densitometry (Scion Image; **Figure 2(C)**). A linear amplification window of approximately 27 - 35 cycles was observed; subsequent assays used 33 cycles, which fell within this linear range.

2.3. Semi-Quantitative RT-PCR and Tissue Distribution

RNA from female tissues (brain, heart, liver, intestine, ovary, skeletal muscle) and male tissues (brain, testis) was extracted by the same procedure and reverse transcribed as above. Semi-quantitative RT-PCR for cyp19a1b and β-actin (internal control) used the conditions in Section 2.2, with gene-specific primers for β-actin. Equal volumes of PCR products were separated on 1.5% agarose/TAE gels, stained with ethidium bromide and imaged with an Olympus digital system. Band intensities were quantified by Scion Image and expressed as cyp19a1b/β-actin ratios. Tissue-specific expression patterns and validation of the labeled probe are shown in **Figure 3**.

2.4. Non-Radioisotopic *in Situ* Hybridization

A DIG-labeled PCR probe for cyp19a1b was generated using the cloned zebrafish cyp19a1b fragment as template and the same primer pair described in Section 2.2. DIG DNA Labeling Mixture (Boehringer Mannheim/Roche Diagnostics,

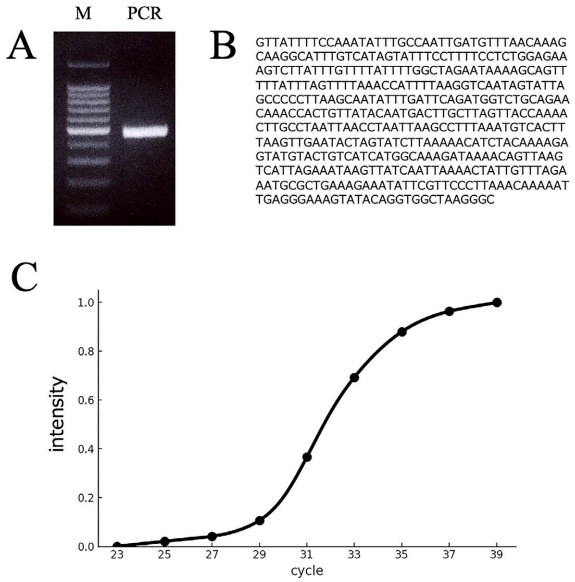

Figure 2. Partial cloning and PCR optimization of zebrafish cyp19a1b. (A) Agarose gel showing a distinct ~500 bp amplicon from female brain cDNA amplified with the cyp19a1b primer pair. M: 100 bp ladder. The product was gel-purified, cloned and sequenced to confirm identity. (B) Nucleotide and deduced amino acid sequences of the ~500 bp cyp19a1b fragment, demonstrating high homology to zebrafish brain aromatase and validating primer specificity used in downstream RT-PCR and probe generation. (C) PCR-cycle optimization for semi-quantitative analysis: densitometric plot of band intensity versus cycle number (23 - 39 cycles) using female brain cDNA. A linear amplification window (~27 - 35 cycles) was identified; subsequent assays used 33 cycles to remain within the linear range for relative quantification.

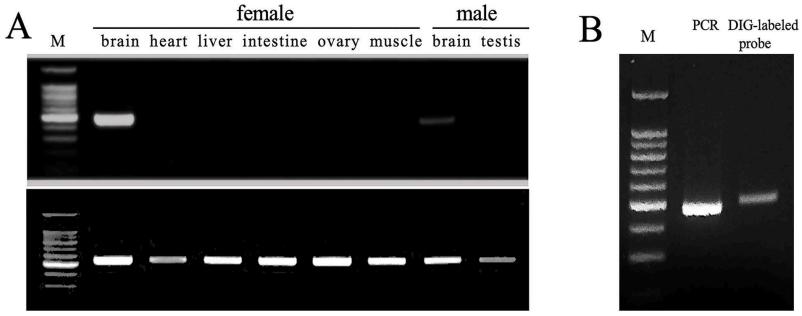

Figure 3. Tissue distribution of cyp19a1b and validation of the DIG-labeled probe. (A) Semi-quantitative RT-PCR of cyp19a1b transcripts in female tissues (brain, heart, liver, intestine, ovary, skeletal muscle) and male tissues (brain, testis). Upper panel: cyp19a1b bands. Strong expression is confined to female brain; male brain shows low basal signal; testis is negative. Lower panel: β-actin internal control. M: 100 bp ladder. (B) Agarose gel showing the conventional cyp19a1b PCR product ("PCR" lane) and the DIG-labeled cyp19a1b probe ("DIG-labeled probe" lane). The DIG-labeled probe migrates slightly more slowly and therefore appears as a band of larger apparent size than the unlabeled ~500 bp fragment, consistent with incorporation of DIG-conjugated nucleotides into the cyp19a1b amplicon. M: 100 bp ladder.

Mannheim, Germany) with Taq DNA polymerase (TOYOBO) was used with cycling at 94˚C 30 s, 50˚C 1 min and 72˚C 1 min for 35 cycles.

Female heads were fixed overnight in 4% paraformaldehyde (PFA) in DEPC-treated water (analytical-grade reagents; FUJIFILM Wako, Osaka, Japan), cryoprotected in 30% sucrose/DEPC (2 - 4 h), embedded and cryosectioned at 10 µm. After drying, sections were post-fixed (4% PFA, 15 min) and pretreated with proteinase K (10 min, room temperature), 0.2 M HCl (10 min), 0.1 M triethanolamine (TEA, pH 8.0; 1 min), and 0.1 M TEA/0.25% acetic anhydride (10 min), then dehydrated (70% - 100% ethanol). Hybridization buffer (50% formamide, 10 mM Tris-HCl pH 7.6, 1× Denhardt's, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA) was preheated (85˚C, 10 min), the denatured DIG probe was applied, and sections were incubated overnight at 37˚C.

Stringency washes were carried out at 42˚C (2× SSC/50% formamide, 30 min; 2× SSC, 20 min; 0.2× SSC, 2× 20 min). Detection used anti-DIG mouse monoclonal antibody (Roche), biotinylated goat anti-mouse IgG and an avidin-biotin complex (ABC) (Vector Laboratories, Burlingame, CA, USA), developed with DAB chromogen. Labeled sections were examined under a light microscope and photographed (Figure 4).

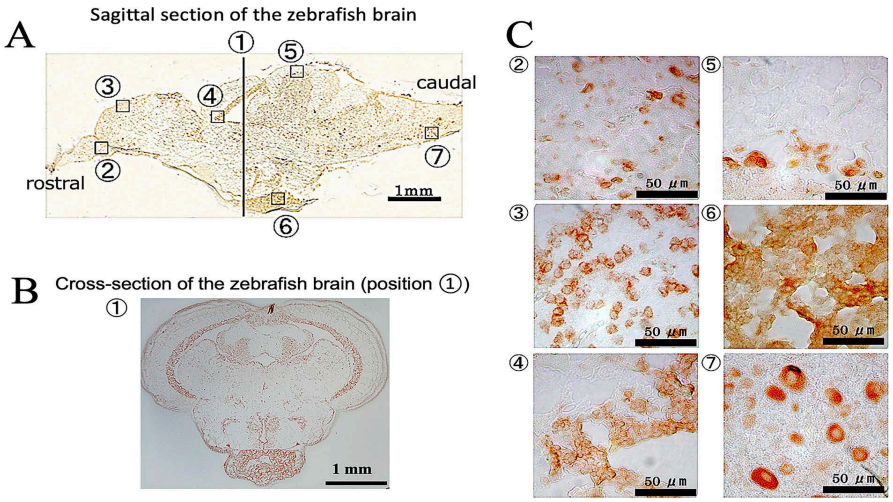

Figure 4. Neuroanatomical localization of cyp19a1b mRNA in female zebrafish brain by non-radioisotopic *in situ* hybridization. (A) Sagittal section of the zebrafish brain processed with a DIG-labeled cyp19a1b probe and DAB chromogen. Boxes ② - ⑦ indicate regions shown at higher magnification in panel C. Rostral and caudal orientations are indicated. (B) Cross-section of the zebrafish brain at position ① in panel A, illustrating the overall pattern of labeling at the tectal level. (C) Higher-magnification views of boxed regions in panel A: ② olfactory lobe with labeled neurons in dorsal/medial territories (glomerular/mitral regions); ③ rostral forebrain (telencephalon) showing scattered positive somata within pallial/subpallial fields; ④ optic tectum highlighting positive neurons in granular layers; ⑤ cerebellum with intense perikaryal labeling of Purkinje cells arranged in a monolayer; ⑥ pituitary gland (adenohypophysis) exhibiting strong cytoplasmic signal in endocrine cells; ⑦ medulla/ventral hindbrain contiguous with the spinal cord, showing scattered labeled neurons. Scale bars: 1 mm in A and B; 50 µm in C.

2.5. Chemical Exposures and Brain Sampling

Adult male fish were exposed for 5 days in glass vessels (one fish per vessel; Figure

1(B)) to 17β-estradiol (E2; 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M) or bisphenol A (BPA; 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M). Dose ranges were selected based on 1) concentrations previously shown to modulate estrogen-responsive genes in fish and other vertebrates, and 2) reported environmentally relevant levels detected in aquatic systems for BPA, thereby allowing assessment of both low-dose and higher-dose effects within a single experimental framework. E2 and BPA (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in analytical-grade ethanol (FUJIFILM Wako, Osaka, Japan) and diluted with fresh water to obtain the desired nominal concentrations. A solvent control group received ethanol alone at the same final concentration as exposure groups, and a water control group received neither ethanol nor test chemicals. Group size was ≥5 fish per condition.

Following exposure, fish were anesthetized with tricaine methane sulfonate (MS-222; Sigma-Aldrich). Brains were rapidly dissected under a stereomicroscope (Figure 1(C), Figure 1(D)), frozen in liquid nitrogen and stored at −80˚C until RNA extraction. Semi-quantitative RT-PCR for cyp19a1b and β-actin was performed as in Section 2.3.

2.6. Data Analysis

Band intensities were quantified by densitometry using Scion Image and normalized as cyp19a1b/β-actin ratios. For each experiment (E2 or BPA), one-way ANOVA was performed across all dose groups, solvent control and water control. When ANOVA indicated a significant main effect, Tukey's honestly significant difference (HSD) test was applied for post-hoc comparisons versus solvent and water control groups. Data are presented as mean ± SD. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Partial Cloning and PCR Optimization

PCR from female brain cDNA yielded a distinct ~500 bp amplicon (Figure 2(A)). Sequence analysis of the cloned fragment showed high identity to zebrafish brain aromatase cyp19a1b, confirming that the primer pair specifically amplified this isoform (Figure 2(B)). Cycle-number titration revealed that densitometric signal increased nearly linearly between ~27 and 35 cycles (Figure 2(C)). On this basis, all subsequent semi-quantitative RT-PCR assays were run for 33 cycles to remain within the linear amplification range.

Thus, we successfully isolated and cloned the zebrafish brain-specific aromatase cDNA, cyp19a1b. This authenticated clone provided an isoform- and species-specific standard that underpinned all subsequent analyses: it served as the template for our semi-quantitative RT-PCR assay and, critically, for synthesis of the DIG-labeled cyp19a1b probe used in the non-radioisotopic *in situ* hybridization that mapped transcript distribution throughout the brain.

3.2. Tissue Distribution and DIG-Labeled Probe Validation

Semi-quantitative RT-PCR demonstrated that cyp19a1b expression was strongly

enriched in female brain (Figure 3(A), upper panel). Heart, liver, intestine, ovary and skeletal muscle showed minimal or undetectable signal. In males, basal cyp19a1b expression in brain was low compared with female brain, and testis was negative. *B*-actin was readily detected in all tissues examined (Figure 3(A), lower panel), confirming RNA integrity and cDNA synthesis.

To validate the DIG-labeled probe used for *in situ* hybridization, we compared the conventional cyp19a1b PCR product with the DIG-labeled PCR product on agarose gel (Figure 3(B)). The DIG-labeled probe migrated slightly more slowly and therefore appeared as a band of larger apparent size than the unlabeled ~500 bp fragment. This upward shift is exactly what is expected when DIG-conjugated nucleotides are incorporated into the DNA strand, and the presence of a single, clearly shifted band indicates that the intended cyp19a1b fragment was efficiently and specifically labeled.

3.3. Neuroanatomical Localization (*in Situ* Hybridization)

Building directly on the cloned zebrafish brain cyp19a1b sequence and its DIG-labeled derivative, we carried out non-radioisotopic *in situ* hybridization to visualize where this aromatase isoform is expressed within the central nervous system. The use of a clone-derived, isoform-specific probe allowed us to interrogate, with high confidence, the genuine spatial distribution of zebrafish brain cyp19a1b transcripts.

Non-radioisotopic *in situ* hybridization using the DIG-labeled cyp19a1b probe revealed widespread expression in the female zebrafish brain (Figure 4). In sagittal sections, positive cells were distributed from rostral to caudal regions, including olfactory bulb, telencephalon, hypothalamus, optic tectum, cerebellum, medulla and pituitary (Figure 4(A)). A cross-section at the level of the optic tectum (Figure 4(B)) highlighted dense labeling in periventricular zones and in discrete neuronal layers.

Higher-magnification views showed that labeled cells in the olfactory lobe, telencephalon and optic tectum had neuronal morphology (Figure 4(C)-②-④). In the cerebellum, large flask-shaped Purkinje cells arranged in a monolayer exhibited intense cytoplasmic staining (Figure 4(C)-⑤). The pituitary gland contained strongly positive endocrine-like cells within the adenohypophysis (Figure 4(C)-⑥). Scattered labeled neurons were also observed in the medulla and ventral hindbrain contiguous with the spinal cord (Figure 4(C)-⑦). Background staining of neuropil was low, and DAB reaction product was largely confined to perikarya and perisomatic regions.

Taken together, these data show that the cloned zebrafish cyp19a1b sequence, when converted into a DIG-labeled probe, provides a powerful and highly specific tool to chart brain aromatase expression at cellular resolution in the adult zebrafish brain.

3.4. E2 Exposure

Semi-quantitative RT-PCR revealed that 5-day E2 exposure increased cyp19a1b expression in adult male brain (Figure 5(A), Figure 5(B)). The ~500 bp cyp19a1b

band intensified with increasing E2 dose, whereas β-actin remained relatively constant. Quantitative analysis of cyp19a1b/β-actin ratios (Figure 5(B)) showed a trend toward dose-dependent up-regulation across 10^{-9} - 10^{-6} M. One-way ANOVA indicated a significant overall effect of treatment. Tukey's HSD test revealed that 10^{-6} M E2 significantly elevated cyp19a1b expression compared with both solvent and water controls ($P < 0.05$ or $P < 0.01$, as indicated by asterisks in Figure 5(B); single asterisk (*) indicates $P < 0.05$ and double asterisk (**) indicates $P < 0.01$ vs. solvent and/or water control. Lower concentrations tended to increase expression but did not reach statistical significance versus solvent and water controls.

3.5. BPA Exposure

BPA exposure produced a non-monotonic (U-shaped) dose-response pattern in male brain cyp19a1b expression (Figure 5(C), Figure 5(D)). RT-PCR gels showed clear cyp19a1b bands across doses of 10^{-8} - 10^{-5}M (Figure 5(C)). Densitometric analysis (Figure 5(D)) demonstrated that 10^{-8} M and 10^{-5} M BPA significantly increased cyp19a1b/β-actin ratios compared with solvent and water controls ($P < 0.05$ or $P < 0.01$), whereas intermediate doses (10^{-7} and 10^{-6} M) did not differ from controls. As in Figure 5(B), a single asterisk (*) denotes $P < 0.05$ and a double asterisk (**) denotes $P < 0.01$ versus solvent and/or water control. Thus, BPA induced cyp19a1b expression at both the lowest and highest doses tested, consistent with non-monotonic low-dose behavior.

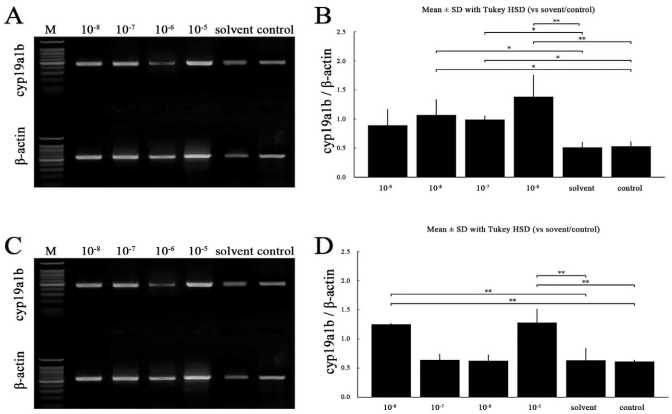

Figure 5. Effects of E2 and BPA on cyp19a1b expression in male zebrafish brain measured by semi-quantitative RT-PCR. (A) Representative RT-PCR gels for cyp19a1b and β-actin following 5-day exposure to E2 (10^{-9} - 10^{-6} M), solvent control (ethanol only) and water control. M: 100 bp ladder. (B) Densitometric quantification of cyp19a1b/β-actin ratios for E2 exposure (mean ± SD; n ≥ 5 fish per group). Data were analyzed by one-way ANOVA followed by Tukey's HSD test versus solvent and water controls. Single asterisk (*) indicates $P < 0.05$; double asterisk (**) indicates $P < 0.01$, compared with solvent and/or water control. (C) Representative RT-PCR gels for cyp19a1b and β-actin following 5-day exposure to BPA (10^{-8} - 10^{-5} M), solvent control and water control. M: 100 bp ladder. (D) Densitometric quantification of cyp19a1b/β-actin ratios for BPA exposure (mean ± SD; n ≥ 5 fish per group). One-way ANOVA followed by Tukey's HSD test revealed significant up-regulation at 10^{-8} M and 10^{-5} M BPA compared with solvent and water controls. Again, (*) denotes $P < 0.05$ and (**) denotes $P < 0.01$ versus solvent and/or water control, indicative of a non-monotonic (U-shaped) dose-response.

4. Discussion

Our molecular and anatomical data establish that zebrafish brain aromatase, cyp19a1b, functions as a sensitive central readout of estrogenicity in adult males. We confirmed by cloning and sequencing that our semiquantitative RT-PCR assay specifically amplifies the zebrafish brain aromatase isoform. Tissue distribution analysis showed that cyp19a1b expression is strongly enriched in the brain, particularly in females, with negligible expression in peripheral tissues and male testis. Furthermore, nonradioisotopic *in situ* hybridization revealed widespread expression of cyp19a1b mRNA throughout the central nervous system and pituitary, including olfactory, forebrain, hypothalamic, tectal, cerebellar, and hindbrain regions. This distribution pattern is consistent with previous reports in teleosts [13] [14] and supports the concept that local neurosteroidogenesis plays important roles in modulating neuronal activity, neurogenesis, and neuroendocrine regulation [15]-[17].

Using semiquantitative RT-PCR, we demonstrated that short-term exposure to 17β-estradiol (E2) induces cyp19a1b expression in the adult male zebrafish brain. In particular, exposure to 10^{-6} M E2 resulted in a statistically significant increase in cyp19a1b expression compared with solvent and water controls, indicating that the male brain retains inducible aromatase capacity despite low basal expression. These findings are in line with earlier studies showing estrogen-dependent regulation of brain aromatase in zebrafish and other teleost species [11] [13] [14].

Notably, exposure to bisphenol A (BPA) elicited a non-monotonic (U-shaped) dose-response pattern, with significant induction of cyp19a1b at both low (10^{-8} M) and high (10^{-5} M) concentrations, while intermediate doses were ineffective. Such non-monotonic dose-response relationships are increasingly recognized as characteristic features of endocrine-disrupting chemicals and challenge traditional toxicological assumptions based on monotonicity [18]. Several mechanisms have been proposed to explain these responses, including differential activation of estrogen receptor subtypes, engagement of membrane-initiated versus nuclear signaling pathways, and feedback regulation within steroidogenic and neuroendocrine circuits [18]. Previous experimental studies on BPA and other endocrine disruptors have demonstrated that low-dose effects can arise through signaling mechanisms distinct from those operating at higher concentrations, lending support to these interpretations.

From a biomarker perspective, cyp19a1b offers features that are complementary to classical indicators of estrogenic exposure in fish, such as vitellogenin. Vitellogenin is a hepatic, female-biased plasma protein that robustly reflects systemic estrogenicity [5], but it primarily reports peripheral endocrine effects. In contrast, brain cyp19a1b directly reflects estrogenic signaling within the central nervous system and is readily inducible even in adult males. Importantly, cyp19a1b responds to very low-dose and non-monotonic estrogenic stimuli that may not always be detected by vitellogenin induction alone. In addition to its high tissue specificity and mechanistic linkage to neuroendocrine regulation, cyp19a1b can

be quantified by relatively simple molecular approaches without the need for plasma sampling. These characteristics make brain cyp19a1b a practical, low-dose-responsive, male-applicable, and CNS-specific biomarker for screening subtle neuroendocrine effects of endocrine-active substances.

The present study nevertheless has several limitations. First, gene expression was assessed by semiquantitative rather than real-time quantitative RT-PCR, which may limit resolution at very low expression levels. Second, nominal exposure concentrations were used without direct analytical verification of BPA levels in the water. Third, the study focused on transcript-level changes without parallel assessment of behavioral, hormonal, or functional neuroendocrine endpoints. Addressing these limitations in future work—by incorporating quantitative PCR, chemical measurements, region-specific analyses, and functional endpoints—will further strengthen the utility of cyp19a1b as a regulatory biomarker.

5. Conclusion

In conclusion, our results demonstrate that 1) cyp19a1b is a brain-enriched, estrogen-responsive gene in zebrafish; 2) adult male brain retains inducible aromatase capacity in response to E2; 3) BPA induces cyp19a1b expression in a non-monotonic manner, with significant effects at both low and high doses; and 4) brain cyp19a1b, measurable by semiquantitative RT-PCR, represents a practical and complementary neuroendocrine biomarker for endocrine-active substance screening and environmental monitoring.

Ethics Approval and Consent to Participate

All animal procedures were approved by the Animal Experimentation Committee of Kyoto Prefectural University (KPU200819R) and conducted in accordance with institutional guidelines.

Availability of Data and Materials

Data supporting the findings are contained within the manuscript and figure legends; underlying gel images and raw densitometry files are available from the corresponding author upon reasonable request.

Authors' Contributions

K.A. conceived and designed the study, performed experiments, analyzed data and drafted the manuscript. N.F. designed the primers for zebrafish cyp19a1b and carried out sequencing of the PCR products to confirm gene identity. Y.T. supervised the project, contributed to study design and interpretation, and critically revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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