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Acute exposure to 4-OH-A, not PCB₁₂₅₄, alters brain aromatase activity but does not adversely affect growth in zebrafish.

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Abstract

Acute developmental exposure to pharmaceuticals or environmental contaminants can have deleterious, long lasting effects. Many of these compounds are endocrine disruptors (EDCs) that target estrogen signaling, with effects on reproductive and non-reproductive tissues. We recently reported that zebrafish larvae transiently exposed to the pharmaceutical EDC 4-OH-A display visual deficits as adults. Here, we examine whether these long-term effects are due to compound-induced morphological and/or cellular changes. Zebrafish aged 24hr, 48hr, 72hr, or 7 days post-fertilization (larvae) or 3–4mos (adults) were exposed to either 4-OH-A or PCB₁₂₅₄ for 24hr. After that time, notochord length, eye diameter, inter-eye distance, and heart rate were measured from larvae; and aromatase (estrogen synthase) activity was measured in homogenates of adult brain tissue. In general, indices of larval growth and development were not altered by 24hr exposure to either compound. 4-OH-A potently inhibited aromatase activity, while PCB₁₂₅₄ did not, with inhibition continuing even after removal from treatment. These results support differential function of EDCs and indicate that developmental exposure to 4-OH-A causes sustained inhibition of aromatase, which could be associated with altered adult behaviors.

Keywords

Estradiol; formestane; Endocrine Disruptors; EDCs; Development; Aroclor1254

1. Introduction

Endocrine disrupting compounds (EDCs) are environmental contaminants (Söfker and Tyler, 2012) that pose risks to human health (Pasqualini and Chetrite, 2006), particularly when exposure occurs during development (Brion et al., 2012; Colciago et al., 2009; Kazeto et al., 2004; Leet et al., 2011; Ryan and Vandenberg, 2006; Si man et al., 2007). Development

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involves the coordinated response to environmental cues, orchestrated cellular signals, and integration of functional circuits; any change or perturbation to one component can lead to adverse anatomical or cellular changes. It is not surprising then, that EDC exposure is most deleterious during prenatal and early postnatal development when organs and neural systems are forming (NIEHS—National Institute of Environmental Health Sciences, 2010); with exposure at these times altering cognitive abilities and learning (Jašarević et al., 2011), affecting perception of environmental cues (Solomon and Schettler, 2000; Solomon and Weiss, 2002), and changing gene expression (Karmaus et al., 2011).

EDCs are human-made or naturally-produced compounds that can mimic or block the action of endogenous hormone(s) and/or alter hormone synthesis (Coe et al., 2010; DeCoster and Van Larebeke, 2012; Söffker and Tyler, 2012; Wingfield and Mukai, 2009). EDCs enter the aquatic environment (Le Page et al., 2011; LePage et al., 2008; Söffker and Tyler, 2012) through improper waste disposal, snowmelt, leaching, or as byproducts of chemical and manufacturing processes (US EPA, OSWER, 2013), whereas exposure in terrestrial animals including humans, may occur mostly through pharmaceuticals and personal care products (Kasprzyk-Hordern et al., 2009, 2008). Environmental EDC concentrations range from ng/L to ug/L (Chen et al., 2007), or ~1000x more concentrated than human hormone levels (Lee et al., 2008; Narayanaswamy et al., 2016), supporting their extreme potency even at relatively low concentrations. As of September 2018, 1,484 EDCs have been identified (The Endocrine Disruption Exchange (TEDEX), 2018): >50% target estrogen pathways and many have reported effects in both humans and animal models (Gore et al., 2015). Most published work has focused on acute exposure or lethal dose 50 (LD50) determination, with changes in growth, survival, and behavior as endpoints (Colciago et al., 2006; Hamad et al., 2007; Houser et al., 2011). While these studies clearly show non-reproductive effects of EDCs, it is unclear precisely how some exert their specific effects at the cellular level or if developmental exposure has lasting effects.

17 β -estradiol (E₂), the biologically active estrogen, is produced endogenously via the enzyme aromatase from the substrate testosterone (Lephart, 1996; Simpson et al., 2002). Estradiol is most commonly known for its role in reproduction, however it is also involved in neuronal cell migration and survival (Grandel et al., 2006), synaptic plasticity (Hamad et al., 2007), cognitive and social development (Nelson and Bulun, 2001), and memory retention (Ryan and Vandenberg, 2006), among several other endpoints. Consequently, exposure to an anti-estrogenic EDC affects many systems and processes. This study focuses on the biochemical and anatomical effects of two reported anti-estrogenic compounds: the polychlorinated biphenyl (PCB) congener PCB₁₂₅₄ (Aroclor₁₂₅₄) and the prescribed pharmaceutical 4-hydroxy-androstendione (4-OH-A or Formestane).

PCBs were developed in the 1940's and used by electrical manufacturing companies until they were banned in the 1970's (Stohs, 2014; U.S. Environmental Protection Agency, 2013) because of proven toxicity to humans and wildlife. Much of the waste products from these manufacturing facilities was dumped into local bodies of water, causing bioaccumulation in the water supply (National Toxicology Program, 2011). Though 40 years have passed (Stohs, 2014; U.S. Environmental Protection Agency, 2013), PCBs remain a global contaminant with > 200 different congeners identified (Mullins et al., 1984). Measurable

PCB levels are present in human blood, adipose tissue, and breast milk (Solomon and Weiss, 2002); these compounds are able to cross the placenta, reduce gestational length and birth weight (Taylor et al., 1984), and affect fetal development (Shen et al., 2007). More than half of the identified congeners affect estrogen signaling (Breivik et al., 2007; Meijer et al., 2003; Wolff, 1997) with antiestrogenic compounds blocking estrogen receptors or inhibiting aromatase and preventing estradiol synthesis.

As a pharmaceutical compound, the activity of 4-OH-A is well known. It is a steroidal aromatase inhibitor that competes with androgens, irreversibly binding to aromatase and blocking estrogen synthesis (Hamad et al., 2007). 4-OH-A is used in the treatment of estrogen-receptor positive breast cancer in postmenopausal women (Dowsett, 1993), because it lowers the amount of estrogen available, slowing the growth of some cancers. 4-OH-A has also been used experimentally, as reports showed significant effect on body length between 111 and 160 hpf following chronic 4-OH-A exposure (Hamad et al., 2007).

Previously, we showed transient (24hr) developmental exposure to 4-OH-A causes deficits in adult visual-guided behaviors (Gould et al., 2017), indicating neuronal effects outside the reproductive axis. We do not know the specific mechanism that caused the altered behaviors; however, we hypothesize two possible causes: (1) compound- induced morphological changes that affect the fish's ability to respond to the stimulus and/or (2) compound-induced changes in cellular/biochemical pathways. Both of these causes could be affected by disruption of estrogen pathways, either individually or simultaneously. Here, we address these possibilities by assessing if a single (24hr) exposure to either 4-OH-A or PCB₁₂₅₄ is sufficient to directly alter aromatase activity and gross morphology. Measurements of anatomical parameters and heart rate were used to identify morphological changes. Changes in estrogen biosynthesis was assessed with the aromatase activity assay (Roselli and Resko, 1991). Given that both 4-OH-A or PCB₁₂₅₄ are reported to inhibit estrogen synthesis (Hamad et al., 2007; Hany et al., 1999) and adversely affect behavior (Gould et al., 2017; Hany et al., 1999), we anticipated significant effects in all treatment groups.

2. Materials and Methods

2.1 Animal Maintenance

Adult wild type zebrafish (*Danio rerio*) were obtained from in-house stocks at the Zebrafish Ecotoxicology, Neuropharmacology, and Vision (ZENV) laboratory at American University and maintained at 28–29°C on a 14-hour light: 10-hour dark photoperiod and fed twice daily. Larvae were obtained from spawning of adults throughout 2015–2016. Following a successful spawn, fertilized eggs were separated, staged, cleaned, and maintained in the laboratory using embryo rearing solution (0.006% instant ocean and 0.01% methylene blue) at the same temperature and photoperiod as the adults until placed in their designated experimental conditions (Kimmel et al., 1995; Lawrence, 2007; Westerfield, 2000). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the ZENV laboratory at American University.

To identify the correct concentration of 4-OH-A and PCB₁₂₅₄ to use in exposure experiments, we first performed a set of *ex vivo* experiments with excised adult brain tissue.

This analysis also used, fadrozole (Roselli and Resko, 1991), a potent and specific aromatase inhibitor (Wade et al., 1995; Villeneuve et al., 2009). Fadrozole (Sigma) was used because it actively and completely inhibits aromatase activity in brain tissues (Roselli and Resko, 1991). For these experiments, adult zebrafish ($n=10\text{--}30/\text{treatment}$) of mixed sex were removed from holding tanks and euthanized with 0.02% tricaine solution. Whole brain was extracted, placed in a microcentrifuge tube, weighed (mg, Sartorius balance), flash frozen (dry ice), and stored at -80°C . Upon thawing, tissue was separated into different treatment groups (Roselli and Resko, 1991): three concentrations of 4-OH-A (10nM, 100 μM , 1mM) and four concentrations of PCB₁₂₅₄ (0.125, 0.5, 1.0, or 2.0mg/L), reflecting the effective range of each compound (Houser et al., 2011), were tested. As a result of this study, 50 μM 4-OH-A and 0.5mg/L PCB₁₂₅₄ were selected as the concentrations to use for subsequent exposure experiments.

2.2 Exposure groups

At 24, 48, 72 hours (h), and/or 7 d (d) post-fertilization (pf) larval zebrafish were transferred to one of four experimental treatments for 24 hours: (1) control (water or 0.004% EtOH or 0.05% MeOH), (2) 4-OH-A (50 μM), (3) PCB₁₂₅₄ (0.5 mg/L). An initial pilot study determined that survival and overall health of animals exposed to 0.004% EtOH (vehicle for 4-OH-A) or 0.05% MeOH (vehicle for PCB₁₂₅₄) were not different from water controls (data not shown), so only the vehicle control was used subsequently. The exposure ages were chosen due to their correspondence with specific events in visual system development (Diotel et al., 2011; Easter and Nicola, 1996; Eisner and Luoh, 2011; Muto et al., 2005; Schmitt and Dowling, 1999) allowing us to unequivocally target the precise developmental stages and times most vulnerable to aromatase inhibition. A subsample of zebrafish larvae at each time point were euthanized immediately after the 24hr exposure with 0.02% tricaine and fixed in 4% paraformaldehyde for anatomical analysis.

A parallel study was also performed with adults, to obtain enough tissue for the aromatase assay. Adults (15/treatment, mixed sex) were placed into temperature-controlled 40L conical aquaria containing one of 3 treatment groups (1) control, (2) 4-OH-A, or (3) PCB₁₂₅₄. After 24hr, subjects were euthanized (0.02% tricaine) and whole brains were collected and pooled (3/group). In addition, to determine if the 24hr exposure period caused long-term inhibition, experiments were performed a second time, collecting tissue both immediately after exposure and after a 3-day washout/recovery period in system water.

2.3 Anatomical assessment

Fixed larvae ($n=4\text{--}8/\text{treatment}/\text{age}$) were photographed using a stereomicroscope (Olympus SXZ16) equipped with an Olympus DP72 color camera and MetaMorph software. ImageJ was used to quantify all gross morphology measurements on imported images. To set the ruler function on ImageJ to scale, a ruler was also placed under the microscope during each imaging session for calibration.

Notochord length was measured as the length of the most anterior part of the head to the most posterior end of the notochord. Eye diameter was measured as the length from the most anterior to the most posterior part of the eye. Inter-eye distance, the left to right length

between the most anterior portions of each eye, was measured from the dorsal side of each fish. Each measurement was made in triplicate and then averaged to reduce the chance of measurement error (Reider and Connaughton, 2014).

For heart rate assessment, 72hpf larvae were exposed to either 4-OH-A or PCB₁₂₅₄ for 24hr. Larvae were then transferred to control conditions for 24hr before heart rate measurement. Since chronic exposure to PCB₁₂₅₄ is reported to adversely affect cardiac development in zebrafish larvae (Li et al., 2014), we waited 24hr after removal from treatment to measure heart rate. Five larvae from each treatment group were selected randomly by gently swirling the petri dish and choosing the first 5 individuals that were picked up with a pipette. These five larvae were embedded in methylcellulose and heart rate was measured under a dissecting microscope for 15sec and then multiplied by 4 in order to obtain beats per minute (bpm). Three measurements from each fish were obtained and averaged to get the most precise heart rate.

2.4 Aromatase activity assay

The assay used in the present study was similar to that described previously (Ankley et al., 2002; Balthazart et al., 2006; Cheshenko et al., 2008; Hinfray et al., 2006; Pedersen et al., 2017) with minor modifications. We optimized original protocols for microsomes (Roselli and Resko, 1991), using homogenates of thawed whole adult zebrafish brains randomly sampled in triplicate (3/tube), with 3 tubes/treatment (2 replicate tubes, 1 assessing non-specific background radioactivity). In brief, each tube received homogenate (50µL), TEK buffer (50µL), 100nM androstenedione (50µL, Perkin Elmer; specific activity=24 Ci/mMol) and 4.8mM NADPH (50µL). Each background tube contained the same solutions except 2mM Fadrozole (50µL) was diluted in the TEK buffer. Control tubes contained TEK buffer (200µL); the recovery tube contained androstenedione (50µL) and TEK buffer (150µL). Homogenate (50µL) was aliquoted to an additional microcentrifuge tube and saved (−80°C) for total protein analysis.

Tubes were vortexed, centrifuged, and incubated (15min) in a 37°C shaking water bath. The reaction was stopped by placing samples on ice and adding 400µL ice-cold 10% trichloroacetic acid with 2% charcoal. Tubes were vortexed and centrifuged (4°C) for 20min at 5,000rcf; supernatant was removed and passed through columns made by Pasteur pipettes plugged with a 3cm glass bead and filled with 1.4mL dowex cation exchange resin. Columns were rinsed 3x with dH₂O and effluent collected in scintillation vials. Ecoscint A (10mL, National Diagnostics) was added and radioactivity counted for 5min after a 4hr incubation on a Beckman LS-6500 scintillation counter. Radioactivity, in femtomoles (fmol) estradiol (E2)/mg tissue/min, was determined by subtracting the disintegrations per minute (dpm) of control samples from the average value of the 2 replicate tubes (Roselli and Resko, 1991). This value was then normalized to the amount of protein/column, determined with a Bradford assay (ThermoFisher).

2.5 Statistical Analysis

Anatomical measurements were compared at each time point using an independent samples t-test (SPSS software, version 25). Control measurements were compared to treatment

morphology measurements using raw data from each. Significance was kept at an alpha of 0.05. Differences across treatment in the aromatase assay experiments were assessed using a 1-way ANOVA ($\alpha=0.05$) followed by a Tukey-Kramer post hoc test, with separate statistical analyses performed on each assay. Since PCB₁₂₅₄ and 4-OH-A used separate controls for the *in vivo* experiments, data from each experiment was analyzed separately using independent students t-test. For the long-term study, data was analyzed using a 2-way ANOVA ($\alpha=0.05$), followed by a Tukey-Kramer post hoc test; time (within factor) and treatment (between factor) were the variables.

3. Results

3.1 Aromatase Activity in excised brains (*ex vivo* study)

Control aromatase activity in excised adult brain tissue averaged 291.6 ± 30.69 fmol E2/mg protein/min (Figure 1). Fadrozole (50 μ M) significantly reduced this activity by 93.4% ($p < 0.01$). When *ex vivo* whole brain homogenate was diluted 1:1 with TEK buffer, mean aromatase activity decreased to 49.4% of the initial value or 147.3 ± 16.54 fmol E2/mg protein/min (Figure 1A), indicating a saturating concentration of androstenedione in the assay. Incubating diluted homogenate in fadrozole, reduced aromatase activity by 66.3%; this reduction was not significant ($p = 0.06$). In all, there was a main inhibitory effect of fadrozole on aromatase activity in whole brain tissue ($p < 0.01$), but no significant difference in between diluted and non-diluted tissue ($p = 0.11$). The reduced inhibition in diluted samples identified the importance of concentrated samples, so subsequent experiments used pooled brain tissue.

Similar to fadrozole, 4-OH-A was an effective aromatase inhibitor in zebrafish brain tissue (Figure 2C). Mean control aromatase activity of 265.1 ± 24.2 fmol E2/mg tissue/min was significantly decreased 71.1% to 76.4 fmol E2/mg/min by 100nM 4-OH-A. Higher concentrations of 4-OH-A significantly decreased aromatase activity by 100% ($p < 0.05$). In contrast, there was no significant change in aromatase activity in *ex vivo* brain samples incubated with PCB₁₂₅₄ (Figure 2B, $p = 0.172$).

3.2 Larval exposures assessed compound-induced anatomical changes

3.2.1 Acute Mortality—Overall survival was high in all groups. Larvae acutely exposed to either 0.5mg/L PCB₁₂₅₄ or 50 μ M 4-OH-A at 24hpf, 48hpf, 72hpf, or 7dpf for 24hr, experienced little to no mortality (<8%) across treatment groups (data not shown).

3.2.2 Notochord Length—There was significant effect of exposure on notochord length at the 48hpf age when treated with 0.5mg/L PCB₁₂₅₄. Larvae in this group were significantly smaller (2.9 ± 0.05 mm) compared to controls (3.1 ± 0.05 mm; $p = 0.031$). No other PCB₁₂₅₄ exposure time point showed a significant difference in body length (Figure 2A). In contrast, for all experimental ages, 4-OH-A treated animals had smaller notochord lengths than controls, though these differences were not statistically significant (Figure 2B).

3.2.3 Eye Diameter—There were no significant differences in eye diameter between control and PCB₁₂₅₄-treated animals at any exposure age (Figure 2C). There was a main

effect of 4-OH-A treatment on eye diameter at 7dpf only, with smaller mean eye diameter measurements ($276 \pm 9.1 \mu\text{m}$) observed compared to controls ($333 \pm 8.9 \mu\text{m}$, Figure 2D). Eye diameter measurements in larvae initially exposed to 4-OH-A at either 24hpf or 48hpf were also smaller than controls; however, these differences were not significant.

3.2.4 Inter-eye Distance—For all experimental ages, PCB₁₂₅₄ treated animals had smaller, though not significant, inter-eye distances than controls (Figure 2E). There was no effect of exposure to 4-OH-A on inter-eye distance in any of the treatment groups (Figure 2F).

3.2.5 Larval Heart Rate—Larval fish treated with PCB₁₂₅₄ for 24hr displayed no change in heart rate when assessed after a 24hr recovery period (Figure 3A), suggesting no short-term deleterious effects of PCB exposure. Larval fish similarly treated with 4-OH-A also showed no effect of compound exposure on heart rate (Figure 3B).

3.3 Adult exposures assessed compound-induced cellular/biochemical changes

PCB₁₂₅₄ (0.5mg/L) did not inhibit aromatase activity (Figure 4A, $p=0.81$) in adult fish after a 24hr exposure in tank water. In fact, overall aromatase activity in this PCB₁₂₅₄ treated group was increased (not significantly) compared to controls. This finding agrees with our *ex vivo* data, suggesting PCB₁₂₅₄ is not a direct inhibitor of aromatase activity. In contrast, 24hr exposure to 50 μM 4-OH-A significantly decreased brain aromatase activity in adult zebrafish (Figure 4B). After a 3-day recovery period, 4-OH-A continued to significantly inhibit brain aromatase activity (Figure 4D), indicating long-lasting inhibitory effects. PCB₁₂₅₄ still had no effect on enzyme activity when measured 3 days after removal from treatment (Figure 4C, $p=0.925$), suggesting no latent effects.

4. Discussion

Previously, we reported that transient developmental exposure to 4-OH-A causes deficits in adult visual-guided behaviors (Gould et al., 2017). In this study, we asked whether these long-lasting behavioral consequences are due to compound-induced anatomical and/or cellular changes. To assess morphological effects, we investigated whether acute exposure had any immediate developmental consequences in larvae after the 24hr exposure period. Exposure ages used were the same as those in the visual behavior study. To assess cellular changes, we measured the ability of 4-OH-A and PCB₁₂₅₄ to manipulate brain estrogen synthesis via the enzyme aromatase. We used 4-OH-A in agreement with previous work (Gould et al., 2017). PCB₁₂₅₄ was selected as a comparison compound because of its environmental relevance, its use in previous studies with other animals, including zebrafish, and its reported contradictory actions on estrogen pathways. Overall our results indicate that transient developmental exposure to either compound did not appear to cause consistent, significant changes in morphological features. However, 4-OH-A, but not PCB₁₂₅₄, potently inhibited aromatase activity in zebrafish brain, an effect that occurred after 24-hours of exposure and lasted at least 3 days. These results suggest that the altered visual behaviors reported in larval zebrafish developmentally exposed to 4-OH-A are due to changes in brain aromatase function/reduced estrogen levels and not anatomical deficits.

4.1 Transient 4-OH-A and PCB₁₂₅₄ exposure does not consistently affect development

Acute exposure of zebrafish larvae to 4-OH-A at 24hpf, 72hpf, or 7 dpf was sufficient to alter visually-guided behavior in adult animals (Gould et al., 2017). These altered behaviors are not likely due to compound-induced anatomical changes, as our results suggest large overall differences in gross morphology did not occur. Notochord lengths were consistent with those reported in the literature (Howe DG, Bradford YM, Conlin T, Eagle AE, Fashena D, Frazer K, Knight J, Mani P, Martin R, Moxon SA, Paddock H, Pich C, Ramachandran S, Ruef BJ, Ruzicka L, Schaper K, Shao X, Singer A, Sprunger B, Van Slyke CE, 2013) and all but one of the measurements were statistically similar to controls. The exception was the reduced eye diameter of 7dpf larvae exposed to 4-OH-A. This group of larvae also displayed reduced optomotor responses as adults (Gould et al., 2017), suggesting that the observed change in eye diameter may be an initial effect of 4-OH-A exposure.

PCB₁₂₅₄ treated larvae similarly showed no adverse effects of acute exposure. Only one significant difference was observed: at 48hpf, treated larvae had significantly smaller notochord length measurements. This result was surprising. Published research shows that zebrafish treated with 0.5 mg/L PCB₁₂₅₄ for 72hr have increased mortality and spinal malformations, pericardial edema, yolk sack edema, and body bending (Ju et al., 2012). Adult ovariectomized rats treated with PCB₁₂₅₄ have bone reabsorption, causing decreased femur weigh and total femur volume (Yilmaz et al., 2006). PCB₁₂₅₄-induced breakdown of collagen in ovariectomized rats may be extrapolated to menopausal women living near PCB₁₂₅₄ contaminated water; a higher risk of developing breast cancer and osteoporosis (Yilmaz et al., 2006) is suggested. No skeletal or cardiac abnormalities were observed in our study, possibly due to the shorter (24hr) exposure time.

At a lower dose of PCB₁₂₅₄ than used here (22µg/L) adult carp displayed decreased overall swimming activity, reduced mean activity, and an increased swimming velocity as they moved. Two weeks after consistent exposure to PCB₁₂₅₄, adult carp appeared to adapt as their behavior returned back to control levels (Schmidt et al., 2005). Though we didn't specifically examine the behavior of fish in the PCB₁₂₅₄ treatment group, we did not observe any obvious behavioral differences in swimming patterns of exposed larvae or adults compared to controls. However, qualitative differences in swimming behavior were noted in 4-OH-A treated larvae. These larvae tended to move less, had a characteristic curved tail, and swam in a corkscrew motion, which were all different compared to control larval movement. This 'listless' phenotype has been previously reported to occur following 2–3 days of exposure to 50µM 4-OHA (Nelson et al., 2008). Our results indicate that the 'listless' phenotype may occur earlier, within 24hr of exposure.

After the 24hr exposure, all larvae were placed into system water and reared to adulthood (3mos of age). There was no effect of either 4-OH-A or PCB₁₂₅₄ on subsequent survival to adulthood, and adults displayed normal swimming behaviors. Thus, transient developmental exposure to 4-OH-A or PCB₁₂₅₄ does not appear to consistently affect growth or survival in zebrafish larvae.

4.2 Only 4-OH-A exposure inhibits aromatase in zebrafish brain

We used the aromatase activity assay to directly measure the impact of compound exposure on a specific estrogen signaling component as it has been used with a variety of species (Balthazart et al., 2001), tissues (Balthazart et al., 2001), and cell lines. Our use of this assay is also unique because it allowed us to (1) directly test if PCB₁₂₅₄ is an aromatase inhibitor and compare efficacy with that of 4-OH-A, (2) focus on brain/neuronal tissue, rather than reproductive, and (3) compare an environmental and a pharmaceutical EDC in the same preparation.

Most studies assessing EDC effects on aromatase/estrogen synthesis use either PCR or mRNA analysis to identify changes in gene expression or antibody labeling to localize enzyme location. These molecular methods only indirectly correlate with enzyme activity; whereas, the aromatase activity assay directly measures estrogen synthesis. The high enzyme activity levels in zebrafish brain identified here correlate with other studies identifying aromatase-expressing radial glial cells and aromatase mRNA transcripts in this tissue (Chiang et al., 2001; Goto-Kazeto et al., 2004; Kishida et al., 2001; Le Page et al., 2011).

PCB₁₂₅₄ is reported to be anti-estrogenic (Colciago et al., 2006; Hany et al., 1999) with some studies suggesting this congener is an aromatase inhibitor (Colciago et al., 2006; Hany et al., 1999). Others indicate it binds directly to androgen receptors (Portigal et al., 2002), acts on ER (Portigal et al., 2002), and/or targets thyroid hormone systems (Winneke et al., 2002). When administered to female rats prior to mating and throughout pregnancy, PCB₁₂₅₄ significantly decreased aromatase activity in the brains of male pups (Hany et al., 1999). Our results indicate PCB₁₂₅₄ is not an aromatase inhibitor in zebrafish brain tissue. Species-specific differences (rodents vs. fish), PCB concentration, and uptake/bioaccumulation are all possible reasons for the different findings. Route and duration of administration are other contributing factors. Compared to chronic, maternally derived exposure in rodents, zebrafish adults were acutely exposed to waterborne PCB₁₂₅₄. Finally, one study reporting PCB₁₂₅₄ as an aromatase inhibitor used cell cultures of rat hypothalamic homogenates (Colciago et al., 2006), a preparation clearly different from ours. The absence of an effect of PCB₁₂₅₄ at the cellular level correlates with the lack of an overall effect on larval growth and development. This suggests that this PCB congener, at the concentrations used, would not adversely affect fish development. It is possible that PCB₁₂₅₄ may alter morphology or modulate estrogen signaling in zebrafish through another mechanism that we did not assess, such as directly interacting with ER and/or androgen receptors, affecting E2 and/or testosterone secretion, or altering expression of *cyp19a1* or ERs (Portigal et al., 2002; Winneke et al., 2002).

4-OH-A is clinically used as cancer therapy (Dowsett, 1993) because it irreversibly binds aromatase, decreasing estradiol formation/estrogen levels (Hamad et al., 2007). Our results indicate this mode of action in human patients is also present in zebrafish. In fact, 4-OH-A is particularly potent at inhibiting zebrafish brain aromatase, regardless of method of administration. When incubated with excised whole brain homogenates for only 24hr, 50μM 4-OH-A is 100% effective. Brain aromatase activity was also 100% inhibited in adult fish immersed in a 4-OH-A solution for 24hr. These results reinforce that pharmaceutical

compounds, such as 4-OH-A, can be taken up directly from the environment (water) and that transient/acute exposure causes significant effects at the cellular level. Select anatomical differences also occur: 50µM 4-OH-A reduces eye diameter when administered to 7dpf larvae and resulting adults display reduced visually-guided behaviors. Notably, the effects of 4-OH-A reported here are outside the reproductive axis and are long-lasting as enzyme activity remained suppressed after removal from treatment and zebrafish adults developmentally exposed to 4-OH-A for 24hr have reduced visually-guided responses (Gould et al., 2017).

5. Conclusion

In sum, our results identify 4-OH-A as a potent aromatase inhibitor in zebrafish brain tissue, suggesting it may be used experimentally to assess the effects of other anti-estrogenic EDCs. PCB₁₂₅₄, however, has no effect on aromatase activity. The disruption of enzymatic function by 4-OH-A likely contributed to the long-lasting behavioral consequences in the adult zebrafish treated acutely at either 72hpf or 7dpf. The effects of 4-OH-A at 72hpf appear to be subtle as no morphological differences were observed in younger larvae and heart rates are normal. In all, our results support differential function of EDCs with developmental/prenatal exposure to 4-OH-A, in particular, causing deleterious, long-term consequences.

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Abbreviations:

EDCs	endocrine disrupting compounds
LD50	lethal dose 50
PCB	polychlorinated biphenyl
4-OH-A	4-hydroxy-androstendione
BPM	beats per minute

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Figure 1. Aromatase activity in zebrafish excised whole brain.

(A) Aromatase activity measured in control and fadrozole treated brain tissue. When tissue was diluted 1:1, the amount of aromatase decreased by half. When fadrozole (50μM) was incubated along with the control tissue there is a 93.4% reduction of aromatase activity. Aromatase activity of the diluted tissue was also decreased by fadrozole, though to a lesser extent. Ex vivo measure of aromatase activity with (B) PCB₁₂₅₄ concentrations ranging 0.125 mg/L to 2.0 mg/L and (C) 4-OH-A concentrations ranging 100 nM to 10 mM. None of the concentrations of PCB₁₂₅₄ tested were able to inhibit aromatase activity however, aromatase activity was significantly reduced with 4-OH-A. Bars with different letters are statistically different from one another. Values reported are mean ± SE.

Figure 2. Morphological parameters of larval zebrafish following acute exposure to either 4-OH-A or PCB₁₂₅₄ at 24 hpf, 48 hpf, or 7 dpf.

(A, B) Notochord length, (C,D) eye diameter, and (E,F) inter-eye distance were measured.

There was a significant difference in notochord length for the 48 hpf initial exposure age group treated with (A) 0.5 mg/L PCB₁₂₅₄ but not for (B) the 50 μM 4-OH-A group.

Age. There were no significant effects of (C) PCB₁₂₅₄ treatment on eye diameter at any age; however, there was a significant difference at 7 dpf in the (D) 50 μM 4-OH-A treated group.

No significant differences in inter-eye distance were observed in either group: (E) 0.5 mg/L PCB₁₂₅₄ or (F) 50 μM 4-OH-A. Significant differences between vehicle and treatment groups are denoted with *. Values reported are mean (in mm or μm) ± SE.

Figure 3. Larval heart rate was not altered by transient compound exposure at 72 hpf. Heart rate (beats per minute) measured one day following 24-hour exposure to either (A) 0.5mg/L PCB₁₂₅₄ or (B) 50 μ M 4-OH-A. Values are mean $\pm$ SE.

Figure 4. Aromatase activity of adult zebrafish brain using in vivo measure following 24-hr exposure and in vivo treatment for 24-hr followed by 3-day washout reveals that PCB₁₂₅₄ has no effect on aromatase activity and 4-OH-A resulted in complete shutdown of aromatase activity.

In vivo aromatase activity measured following short term (24hr) exposure with either (A) 0.5 mg/L PCB₁₂₅₄ or (B) or 50 μ M 4-OH-A in aquarium water. Under these conditions, PCB₁₂₅₄ did not inhibit aromatase activity. In contrast 4-OH-A significantly blocked aromatase activity (denoted with *). In vivo aromatase activity after a 3-day washout period was measured in either (C) 0.5 mg/L PCB₁₂₅₄ or (D) 50 μ M 4-OH-A treated animals. Aromatase levels were not significantly changed as a result of PCB exposure. However, aromatase levels were completely suppressed after 24hr exposure to 4-OH-A, a condition that remained even after 3 days removed from treatment. Bars with different letters are statistically different from one another.