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Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish

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ABSTRACT

Developmental bisphenol A (BPA) exposure has been implicated in adverse behavior and learning deficits. The mode of action underlying these effects is unclear. The objectives of this study were to identify whether low-dose, developmental BPA exposure affects larval zebrafish locomotor behavior and whether learning deficits occur in adults exposed during development. Two control compounds, 17β-estradiol (an estrogen receptor ligand) and GSK4716 (a synthetic estrogen-related receptor gamma ligand), were included. Larval toxicity assays were used to determine appropriate BPA, 17β-estradiol, and GSK4716 concentrations for behavior testing. BPA tissue uptake was analyzed using HPLC and lower doses were extrapolated using a linear regression analysis. Larval behavior tests were conducted using a ViewPoint Zebrabox. Adult learning tests were conducted using a custom-built T-maze. BPA exposure to <30 μM was non-teratogenic. Neurodevelopmental BPA exposure to 0.01, 0.1, or 1 μM led to larval hyperactivity or learning deficits in adult zebrafish. Exposure to 0.1 μM 17β-estradiol or GSK4716 also led to larval hyperactivity. This study demonstrates the efficacy of using the zebrafish model for studying the neurobehavioral effects of low-dose developmental BPA exposure.

1. Introduction

There is emerging concern that the environmental contaminant, bisphenol A (BPA), may adversely impact brain development, leading to the National Toxicology Program's recent recommendation to support studies addressing whether BPA affects brain development and behavior of children exposed prenatally or during early childhood to environmentally relevant levels (NTP, 2008). BPA is the primary ingredient used to manufacture polycarbonate and resin-lined food containers. It is also used to make polyvinyl chloride (PVC), some dental sealants, and thermal printing paper used for cash register receipts (Biedermann et al., 2010; Welshons et al., 2006). Large-scale production and incorporation of BPA into numerous consumer products has resulted in exposures to the extent that low BPA levels in the average range of 1–10 ng/ml (i.e., 0.004–0.04 μM) are detectable throughout the United States population (Vandenberg et al., 2010). Although a recent study found that BPA is rapidly metabolized in adults, leading to lower exposure levels than previously thought (Teeguarden et al., 2011), it did not take into account metabolic features prominent in infants and the placenta (e.g., sulfonation and de-glucuronidation, respectively; reviewed in Ginsberg and Rice, 2009). BPA easily crosses the placenta (Takahashi and Oishi, 2000) and breast fed infants are estimated to ingest 0.2–1 μg/kg/day, while infants fed with BPA-containing bottles are estimated to ingest 1–11 μg/kg/day (NTP, 2008). Strikingly, levels as high as 100 μg/kg have been detected in human placenta (Schonfelder et al., 2002). Taken together, these studies describe low, but ubiquitous exposure to BPA and highlight a potential risk to our most vulnerable populations. It is therefore important to confirm whether low-dose BPA exposure impairs central nervous system (CNS) development and behavior and identify the molecular events underlying these effects.

Abbreviations: hpf, hours post fertilization; dpf, days post fertilization; BPA, bisphenol A; ER, estrogen receptor; ERR, estrogen-related receptor; CNS, central nervous system; E2, 17β-estradiol; GPER, G-protein coupled estrogen receptor; HPLC, high-performance liquid chromatography; MO, antisense oligonucleotide morpholino; PVC, polyvinyl chloride; DMSO, dimethyl sulfoxide.

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While studies provide evidence that developmental BPA exposure can result in abnormal sex-specific behavior (Negishi et al., 2004; Palanza et al., 2008), the mode of action by which BPA impacts development is not well understood. BPA is generally characterized as an estrogen disruptor (Ben-Jonathan and Steinmetz, 1998). While typical estrogen disruptor target organs comprise the reproductive system, the brain is also highly dependent on estrogen for proper development (reviewed in Daniel, 2006). Estrogen's classical mode of action is via activation of the nuclear estrogen receptors (ERs), ERα and ERβ. In addition to these nuclear receptors, estrogen-related receptor gamma (ERRγ) and the G-protein coupled estrogen receptor (GPER) have been proposed as potential mediators of BPA-associated estrogen disruption (Okada et al., 2008; Thomas and Dong, 2006).

Human *in vitro* binding studies show that ERRγ, one of three orphan nuclear estrogen-related receptors that activate transcription at estrogen response elements (ERE's) (Tremblay and Giguere, 2007), exhibits strong binding affinity for BPA (Okada et al., 2008). ERRγ is highly expressed in the brain, placenta, kidney, pancreas, and heart during critical periods of human fetal development as determined by mRNA quantification (Heard et al., 2000). ERRγ and other family members play a role in energy homeostasis, and misexpression of ERRγ has been implicated in mouse models of obesity, diabetes, and heart function, and human *in vitro* cancer studies (Alaynick et al., 2007; Ijichi et al., 2011; Rangwala et al., 2010). Despite confirmation of its high expression in the developing mouse and zebrafish CNS (Bardet et al., 2004; Hermans-Borgmeyer et al., 2000; Qin et al., 2007), the role of ERRγ in facilitating performance on tests of neurobehavioral function has not been investigated. The strong binding affinity of ERRγ for BPA coupled with high ERRγ expression in the developing brain supports the hypothesis that BPA exerts its effects on the developing CNS through ERRγ, possibly through crosstalk with ER signaling. This hypothesis can be investigated by employing the classical ER ligand, 17β-estradiol, and a less commonly used synthetic ERRβ/γ ligand, GSK4716.

Although rodent models are being used to investigate the potential mode of action by which developmental BPA exposure elicits neurobehavioral effects, the zebrafish model has yet to be employed to address this gap. The larval zebrafish model offers notable advantages for conducting mode-of-action studies, including external dosing and availability of antisense oligonucleotide morpholinos (MOs) used for transiently knocking down proteins of interest during development. These advantages, coupled with the fact that ERs and ERRs are expressed in the CNS during embryonic zebrafish development (Bardet et al., 2004; Tingaud-Sequeira et al., 2004) and the availability of established larval and adult behavior tests, can be capitalized upon to complement rodent studies (Levin and Cerruti, 2009). The aim of this study was to determine whether relevant doses of BPA during development impact zebrafish neurobehavior and establish a testing paradigm to be used in subsequent mode-of-action studies investigating the roles of ERs and ERRγ.

2. Methods

2.1. Zebrafish rearing

Adult tropical 5D strain (wildtype) zebrafish were raised at the Sinnhuber Aquatic Research Laboratory (SARL) in the Aquatic Biomedical Models Facility Core of the Environmental Health Sciences Center at Oregon State University under standard conditions (28 °C, 14 h light/10 h dark cycle) on a recirculating water system. Embryos obtained from group spawns were washed, screened for viability, and incubated in embryo medium (Westerfield, 2000) at 28 °C. The uptake, toxicity assay, and larval behavior experiments were all conducted at 28 °C and completed by 5 days post fertilization (dpf), at which point larvae were euthanized using MS-222 (tricaine). Larvae destined for adult behavior testing were exposed to 0.1 or 1 μM BPA from 8-120 h post fertilization (hpf), then removed from the exposure solution, thoroughly rinsed with water, and raised at the SARL under standard conditions until approximately 3 months of age, at which time they were shipped overnight to the Neurobehavioral Toxicology Facility, Children's Environmental Health Sciences Center, University of Wisconsin-Milwaukee, where they were raised under standard conditions prior to adult testing. Zebrafish husbandry and behavior testing was conducted in compliance with approved Oregon State University and University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee protocols.

2.2. Chemical preparation

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane; 99% purity, Tokyo Chemical Industry America (TCI), Portland, OR), GSK4716 (4-Hydroxy-2-[(1*E*)-[4-(1-methylethyl)phenyl]methylene]hydrazide; Tocris Bioscience, Ellisville, MO, USA), and 17β-estradiol (E2; Sigma) were dissolved in dimethyl sulfoxide (DMSO; Sigma). BPA stock concentration was confirmed by high-performance liquid chromatography (HPLC) analysis. Exposure solutions were prepared by diluting working stocks in buffered embryo medium at a final vehicle concentration of 0.1% DMSO.

2.3. Larval toxicity assay

The BPA, E2, and GSK4716 toxicity assays were identical to that described in detail in Truong et al. (2011), with the exception that chorions were not removed, embryos were loaded into the exposure plate prior to addition of the exposure solution, and the 24 hpf evaluation was omitted. Briefly, embryos (8 hpf) were placed individually in the wells of polystyrene 96-well plates (Becton Dickinson, NJ, USA) to which 100 μl exposure solution was added. Plates were sealed in parafilm and covered in aluminum foil throughout the 5 day exposure (see Table 1 for a summary and justification of the exposure durations used in this study). After an initial range-finding pilot assay, exposure concentrations were selected to include concentrations causing abnormalities in 100% of the larvae (EC_{100}) at 5 dpf. The concentration ranges selected for the concentration–response curves were BPA: 10–70 μM; E2: 0.1–25 μM; and GSK4716: 0.1–30 μM. Larvae were evaluated for any abnormality or mortality at 5 dpf following 5 days static waterborne exposure. Mortality was recorded as number dead divided by total number of exposed embryos (BPA: $n = 12$ embryos; E2 and GSK: $n = 8$ embryos). Abnormality was calculated as percentage of survivors exhibiting any of the following morphological defects at 120 hpf: yolk sac edema; pericardial edema; craniofacial abnormalities (snout, jaw, eye, otolith, brain); curved body axis; abnormalities in circulation, pigmentation, pectoral fin, caudal fin, or swim bladder; inability to respond to a light touch; or delayed hatching. Percentage of affected embryos per treatment group was plotted against log(exposure concentration) and a sigmoidal non-linear regression curve (log(agonist) versus normalized response) was derived using GraphPad Prism 5.01 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was conducted using SigmaPlot 11.0 (Systat Software, Inc., Chicago, IL, USA). A one-way ANOVA on ranks (Kruskal–Wallis; because data did not pass a Shapiro–Wilk test for normality) followed by a Student–Newman–Keuls post-test was used to determine significant effects compared to controls ($p < 0.05$). Biological replicates were conducted on different days (BPA: $n = 6$ days; E2 and GSK: $n = 5$ days). Representative photos of 5 dpf larvae from which chorions had been removed by enzymatic digestion (pronase) (see Truong et al. (2011) for detailed description) at 6 hpf were taken

Table 1
Summary of exposure durations used in this study.

Assay	Start of exposure	End of exposure	Justification
Toxicity	8 hpf	120 hpf	Standard toxicity assay that encompasses entire organogenesis period
Larval behavior	8–10 hpf (note: larvae exposed from 8 to 58 hpf exhibit behavioral effects identical to those exposed from 10 to 58 hpf)	58 hpf	Window of exposure that leads to behavioral effects (narrow exposure window provides target organ clues); encompasses peak CNS development
Uptake/tissue dose	8 hpf	58 hpf	Intended to closely match the larval behavior test exposure
Adult behavior	8 hpf	120 hpf	Encompasses entire organogenesis period; intended to maximize chance of detecting behavioral effects in adults

using an Infinity2 digital CCD camera (Lumenera Corporation, Ontario, Canada) attached to an Olympus SZ61 stereomicroscope (Olympus America, Inc., Center Valley, PA, USA).

Although chorion removal was only employed for the purpose of obtaining photos, an additional experiment was included to determine whether the presence of the outer protective chorion impacts the effects of BPA exposure. On each of three days (i.e., 3 biological replicates), embryos from pooled group spawns were divided into two groups, one from which chorions were removed at 6 hpf using pronase enzymatic digestion. The embryos ($n = 8$) were then subjected to the 5 day static waterborne exposure and abnormalities were assessed at 120 hpf as described above. BPA concentrations tested were 0, 10, 20, 40, 80, and 100 μM. Percent age of larvae exhibiting any abnormality was plotted as above and significant difference between larvae with chorions intact versus those with chorions removed was determined for each concentration by unpaired *T*-test using SigmaPlot 11.0 ($p < 0.05$).

2.4. Uptake experiment

2.4.1. Experimental design

Eight hpf embryos were exposed in 96-well plates as described above to vehicle (0.1% DMSO), 1, 10, or 100 μM BPA. Following 48 h static exposure, embryos were thoroughly washed using a Biotek ELx50 plate washer (BioTek, Winooski, VT, USA) programmed for 10 washes with 100 μl volume exchange per wash. Next, pools of 50 embryos were collected in polypropylene 1.5 ml safe-lock microcentrifuge tubes (Eppendorf, Hamburg, Germany), 500 μl of reverse osmosis water was added to each tube and the mass of embryos + water was recorded at room temperature (RT). Embryos were homogenized using approximately 160 mg 0.5 mm zirconium oxide beads (Next Advance Inc., Averill Park, NY, USA) and a Bullet Blender® (Next Advance Inc.) set at speed 8 for 3 min. Following 15 min incubation at RT, samples were centrifuged at 18,000 rcf then 400 μl supernatant was collected into 4 ml amber glass HPLC vials and frozen at −20 °C until HPLC analysis. Spiked samples ($n = 9$) for all concentrations tested were quantified to determine extraction efficiency (1 μM = 47 ± 6%, 10 μM = 42 ± 3%, 100 μM = 41 ± 2%). This efficiency was used to calculate BPA tissue concentration in experimentally exposed embryos. Spiked samples were also used to determine the empirical value of the stock solution. Three (1 μM) or 6 (10 and 100 μM) replicate samples per treatment were used for the calculations. A linear regression analysis (GraphPad Prism 5.01) of log-transformed data was used to extrapolate predicted tissue dose at exposure concentrations 0.001, 0.01, and 0.1 μM. Several measures were taken to limit and/or detect potential BPA contamination from unintended sources. First, no polycarbonate plastic was used during or after the exposures; exposure plates were polystyrene and all solutions were prepared in glass. Additionally, DMSO controls were included to rule out potential contamination from unintended sources during the experiment. No BPA was detected in any of these controls. The limit of detection was 0.061 μg/ml (0.267 μM). Reagent blanks (reverse osmosis water only) were included as additional quality controls. No BPA was detected in these blanks.

2.4.2. HPLC analysis

A portion of the BPA stock powder used for embryonic exposures was prepared in methanol (Fischer, HPLC grade) as the HPLC standard. The standard curve consisted of the following 5 points (μg/ml): 0.095, 0.480, 0.950, 4.760, and 9.520 (0.416, 2.103, 4.161, 20.851, and 41.701 μM). Linear fit: Area = 43.0534823 × AMT + 1.2508553; correlation = 0.99981. All calibration points equaled weight. Samples were thawed and vortexed prior to analysis. HPLC analysis was performed on an Agilent 1100 instrument using the following conditions: Column Phenomenex EnviroSep PP column, 125 mm × 4.6 mm, mobile phase (gradient) was Fisher HPLC grade acetonitrile and water. The gradient started at 30% acetonitrile, by 6 min was at 100%, after which it was held at 100% for 1.5 min. The flow rate was 2 ml/min, injection volume was 10 μl. Detection was by fluorescence with excitation at 225 nm and emission at 350 nm.

2.5. Larval behavior assessments

Embryos were exposed as described for 48 h beginning at 8–10 hpf to BPA (0.001, 0.01, 0.1, 1, or 10 μM), E2 or GSK4716 (0.1 μM), or 0.1% DMSO control. (Note: pilot experiments (data not shown) determined that exposure start times of 8 versus 10 hpf resulted in the same outcome on the BPA, E2, and GSK4716 larval behavior tests. Embryos from this age range were selected in order to obtain the desired number of replicates for the trials). All treatments for each compound were equally represented on each 96-well plate. At 58 hpf, embryos were washed using a Biotek plate washer (10 cycles with 100 μl volume exchange), then resealed and incubated at 28 °C in the dark until 5 dpf. On day five, the 96-well plate containing the embryos was placed in a ViewPoint Zebrabox behavior testing system (Viewpoint Life Sciences, Inc., France) in a room with an ambient temperature of 28 °C and their locomotor activity was recorded using the quantization setting (Videotrack V3 software) for 5 min in the dark following a 20 min rest period in the light (light intensity was 100%). This setting utilizes the number of pixels displaced during the 1/30th of a second between two consecutive frames (30 frames per second) to obtain movement of the subject. A difference of 5 or more pixels between two frames was set as the activity threshold. Larvae exhibiting any morphological defects were subsequently removed from the analysis. Duration of activity in seconds per minute was recorded and the sum of activity over the 5 min in the dark was calculated for each larva (i.e., total seconds spent moving above the threshold during the 5 min). Behavior data was collected from 3 (BPA concentration–response) or 4 (BPA versus E2 and GSK4716) groups of 16 larvae. Each group for a given treatment was spawned and therefore tested on a different day. A one-way ANOVA ($p < 0.05$) was used to confirm that there was no statistical difference in activity in the dark among DMSO controls on the different days, after which all morphologically normal larvae from the different testing days for the BPA concentration response ($n = 42–47$) and BPA versus E2 and GSK4716 ($n = 53-59$) were pooled for a single analysis of mean activity for each of the two experiments (SigmaPlot 11.0). Statistical significance for the BPA concentration response was determined by one-way ANOVA, Student–Newman–Keuls post-test, ($p < 0.05$). Since the BPA versus E2 and GSK4716 data did not pass a normality test (Shapiro–Wilk), significance was determined by one-way ANOVA on ranks (Kruskal–Wallis), Dunn's post-test, ($p < 0.05$).

2.6. Adult learning

2.6.1. Adult testing apparatus (T-maze)

Adult zebrafish learning was assessed using a custom-built T-maze constructed of opaque PVC plastic (Fig. 4). The apparatus had a 50 cm long raceway, which included a 10 cm^3 mesh-covered drain area and a 10 cm^3 pre-trial holding chamber. At the end of the raceway were two 20 cm long arms, one to the left and the other to the right. Depth of maze was 15 cm at the drain and 13 cm at the end of each arm; this slope allowed for proper water drainage. To create a level test surface, a perforated shim was inserted so that the water depth throughout the maze was 10 cm. The left arm of the apparatus was differentiated from the other by masking it with alternating stripes of black and white tape. Six electrodes were attached to each arm, three on each side of the arm. A computer-controlled mild electrical shock (2.0 v, 7.7 mA; 200 ms ON, 800 ms OFF for ≤20 s until fish vacated the arm) was delivered through these electrodes 1 s after the fish entered the incorrect arm. Computer-controlled doors at the junction of each arm were installed to prevent the fish from entering the opposing arm once an arm had been selected (as determined by breaking a set of IR beams and corresponding detectors). Soldered electrical contacts were covered with polyurethane to prevent corrosion and potential contamination of the water in the testing chamber.

2.6.2. First reversal learning task

Larvae destined for adult behavior testing were exposed to 0.1% DMSO, 0.1 or 1 μM BPA from 8–120 hpf, then raised as described under standard conditions. A total of 10 fish (five males and five females; eight month olds) per treatment were used for the learning task. Selection of fish to be tested on any given day was randomized between treatments and sexes, and the test administrator was blinded. Each fish was placed in a holding chamber at one end of the raceway until a trial began. At the start of each trial, the door of the holding chamber was manually opened allowing the fish to swim freely down the raceway until it entered either of the two arms. If a fish did not exit the start box within 10 s, then it was gently prodded to initiate the trial. Once a fish made its first selection, this arm was designated the "incorrect" side and the fish would receive a mild shock each time it entered that arm, up to 90 repeated trials (30 trials/day for maximum three days). Each trial ended either when the fish made a correct choice, made an incorrect choice and the 20 s shocking period passed, or made no choice within 1 min. Following each trial, the fish was returned to the holding chamber for 1 min prior to the start of a new trial. The test ended when a fish correctly selected the non-shocking arm for three consecutive trials. For any trial that a fish refused to make a choice (i.e., remained in the raceway or just outside a door, the trial did not receive a score and the record of consecutive correct choices was reset at zero. Each trial was set as a minimum of 20 s (i.e., immediate entrance into either arm) and a maximum of 60 s (e.g., remaining at door but not entering). Fish that did not successfully enter into the non-shocking arm three consecutive times were scored the maximum number of trials performed (i.e., 90). Statistical significance for the first reversal task was determined by two-way ANOVA (sex × treatment), Student–Newman–Keuls post-test ($p < 0.001$) for males and females considered separately. A one-way ANOVA, Student–Newman–Keuls post-test ($p < 0.001$) was used to analyze the combined group (SPSS 13.0).

3. Results

3.1. Developmental BPA exposure <30 μM is non-teratogenic

By conducting a 5 day toxicity assay routinely used in our laboratory, we found no statistical difference in abnormalities in groups of embryos exposed with chorions intact versus chorions removed (Fig. 1A). Subsequent experiments using chorion intact embryos determined that high concentrations of BPA (>70 μM) were required to produce embryonic mortality, and concentrations <30 μM did not produce any apparent abnormalities (Fig. 1B; Note: data points (x-axis) are plotted at the actual exposure concentrations, which were adjusted following quantification of stock

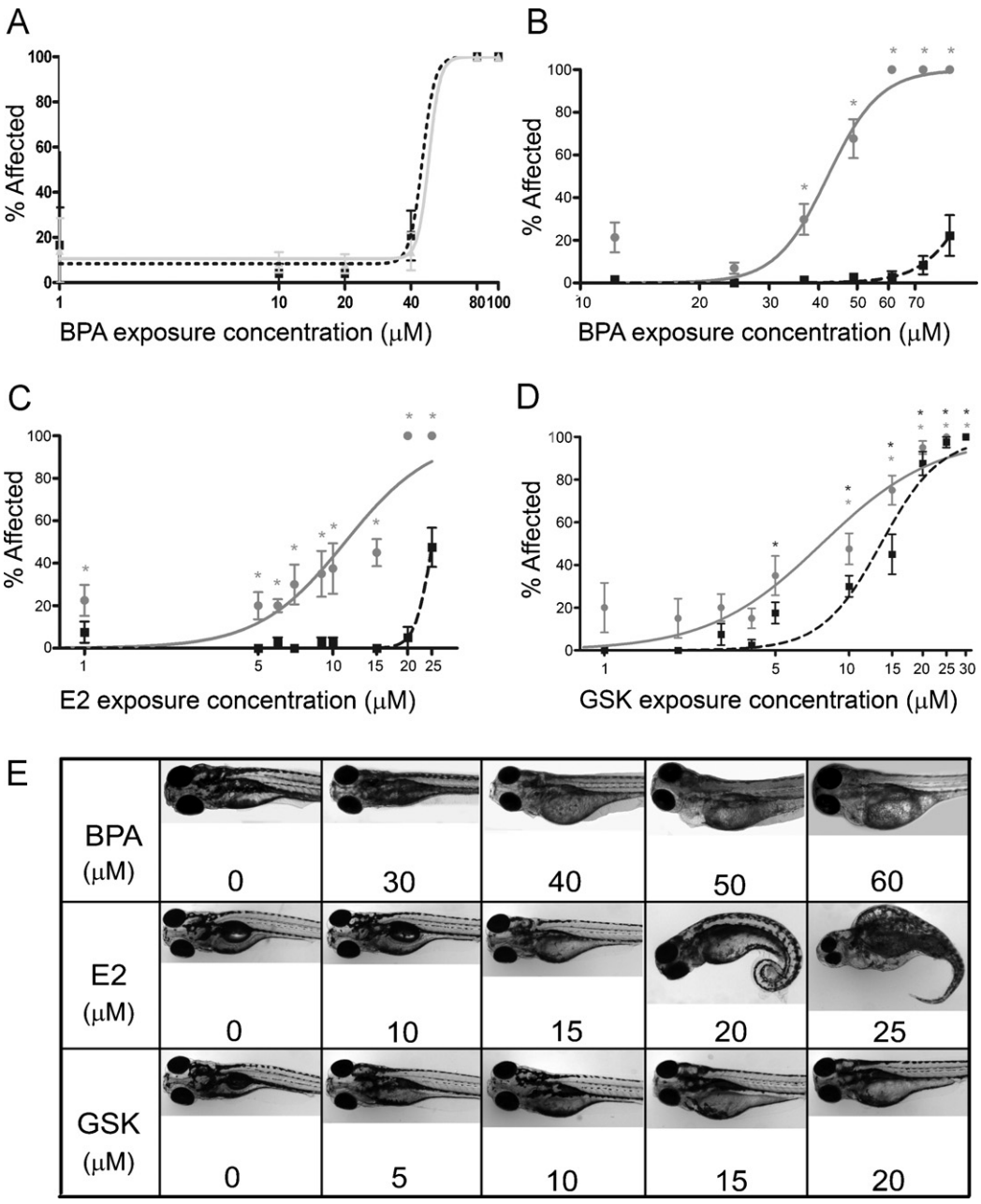

Fig. 1. Morbidity/mortality following developmental exposure to BPA (A and B), E2 (C), or GSK4716 (D). (A) Percentage of chorion intact (dotted black line) versus chorion removed (solid grey line) larvae exhibiting abnormalities following static waterborne exposure to BPA (10–100 μM). Statistical significance determined by unpaired *T*-test for each concentration ($p < 0.05$). Error bars depict SEM. (B–D) Percentage of larvae exhibiting abnormalities (grey circles) or percent mortality (black squares) following static waterborne exposure to BPA (10–70 μM; note: data points (*x*-axis) are plotted at the actual exposure concentrations, which were adjusted following quantification of stock concentration and were slightly higher than expected; e.g., "20 μM" exposure was actually 24.46 μM) (B), E2 (0.1–25 μM) (C), or GSK4716 (0.1–30 μM) (D) from 8-120 hpf. Plot shows % affected versus log(exposure concentration) ±SEM (BPA: $n = 6$, 12 embryos per group; E2 and GSK4716: $n = 5$, 8 embryos per group). Curves were fit by a non-linear regression dose–response analysis. Note: controls for BPA were 12.8% (abnormality) and 0% (mortality); controls for E2 and GSK4716 were 2.5% (abnormality) and 0% (mortality). Statistical significance determined by one-way ANOVA on ranks (Kruskal–Wallis), Student–Newman–Keuls post-test compared to vehicle-exposed embryos, $^{*}p < 0.05$. Error bars depict SEM. (E) Representative photos of 5 dpf larvae showing physical effects of BPA, E2, or GSK4716 exposure. Numbers depict exposure concentrations (μM). Most common observed effects were yolk sac edema, pericardial edema, craniofacial abnormalities, or axis defects.

concentration and were slightly higher than expected; e.g., "20 μM" exposure was actually 24.46 μM). The most common morphological defects associated with BPA exposure concentrations between 30 and 70 μM were yolk sac edema, pericardial edema, craniofacial abnormalities (Fig. 1E), and delayed hatching (not shown). In the embryos exposed to E2, yolk sac edema was the primary effect associated with exposure concentrations between 1 and 15 μM (Fig. 1C and E). Higher E2 exposure concentrations caused more severe yolk sac edema, a characteristic axis curvature, and craniofacial abnormalities. Additionally, GSK4716 (a synthetic ERRβ/γ agonist) was found to be quite potent, with concentrations ≥5 μM causing significant mortality (Fig. 1D). The primary abnormality observed in survivors was yolk sac edema, with some craniofacial abnormalities observed at the higher concentrations (Fig. 1E).

3.2. Embryonic tissue dose is less than waterborne exposure levels

To more directly correlate the BPA exposure concentrations to tissue dose during peak brain development, 8 hpf embryos were exposed to BPA and tissue concentration was sampled from pooled

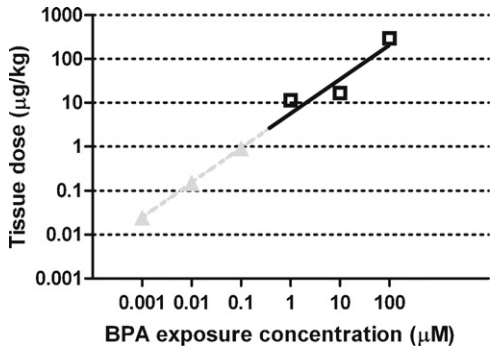

Fig. 2. Tissue dose associated with waterborne BPA exposure. BPA concentration was derived from whole body homogenates (50 pooled embryos) by HPLC following 8–58 hpf static exposure. Black squares are measured values (mean ±SEM, $n = 6$ or $n = 3$ (1 µM)); grey triangles were derived by fitting a linear regression curve to the measured values. Values are plotted as log(tissue dose) versus log(exposure concentration). Figure shows log-transformed data plotted on log-scale axes. Error bars (not visible due to data transformation to a log scale) depict SEM.

embryos at 58 hpf. The 1, 10, and 100 µM exposure concentrations resulted in nominal tissue doses of 12, 17, and 298 µg/kg, respectively (Fig. 2). A linear regression curve was used to calculate tissue dose at exposure concentrations too low to measure; expected tissue doses of 0.02, 0.15, and 0.92 µg/kg were extrapolated following exposure to 0.001 µM (0.228 µg/L), 0.01 µM (2.28 µg/L), and 0.1 µM (22.8 µg/L), respectively.

3.3. Low-dose developmental BPA exposure results in larval hyperactivity

BPA concentrations that did not produce discernable abnormalities and were within relevant ranges for human exposure levels based on the tissue dose experiment (i.e., ≤10 µM; note: this exposure concentration resulted in <100 µg/kg tissue dose, which is the upper level detected in human placenta (Schonfelder et al., 2002)) were selected for behavior testing in order to derive a concentration–response curve. Transient exposure to 0.01 and 0.1 µM BPA resulted in significant larval hyperactivity, whereas the higher concentrations, 1 and 10 µM, did not affect activity (Fig. 3B). To compare the behavioral effects of developmental BPA exposure to the effects of an ER ligand, E2, and an ERRβ/γ agonist, GSK4716, embryos were next exposed to 0.1 µM E2 or GSK4716 and the larval locomotor assay was repeated. Both E2 and GSK4716 also caused significant hyperactivity (Fig. 3C).

3.4. Low-dose developmental BPA exposure results in learning deficits

Mature zebrafish that were transiently exposed to 0.1 or 1 µM BPA from 8–120 hpf required significantly more trials than controls ($F(2, 24) = 13.34$; $p = 0.0001$) to learn to select the correct arm of a T-maze in order to avoid an electric shock (first reversal task) (Fig. 5A and B). There was no significant interaction between sex and BPA treatment ($p = .4798$), but since we hypothesized different effects in males compared to females, we analyzed individual sexes independently. While males exposed to 0.1 µM BPA took significantly longer than control fish to learn the task (74 trials for exposed versus 23 trials for controls, $p = 0.003$), 0.1 µM exposed females did not differ from controls (59 trials for exposed versus 31 trials for controls, $p = .13$) (Fig. 5A). However, both males (79 versus 23 trials, $p = .002$) and females (87 versus 31 trials, $p = .007$) exposed to 1 µM BPA exhibited significant learning delays compared to sex-matched control groups. There was no statistical difference between the 0.1 and 1 µM BPA treatment groups for either sex. When the sexes were pooled, there was a significant effect of treatment on the number of trials taken to choose the correct arm ($F(2,27) = 14.12$, $p < 0.0001$, Fig. 5B). Fish exposed to either of the two BPA concentrations as compared to the control group required significantly more trials to learn (Fig. 5B).

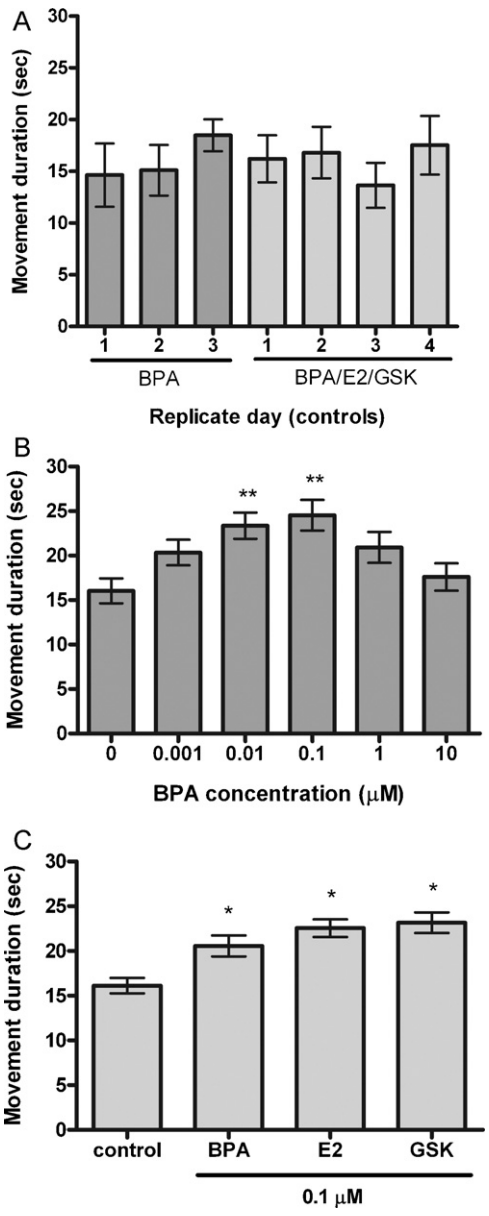

Fig. 3. Locomotor activity of 5 dpf larvae following exposure to 0.1% DMSO (A), BPA (B), E2 or GSK4716 (C) during neurogenesis. (A) Comparison of control larvae on each of the days used for the BPA concentration–response (B) and BPA/E2/GSK4716 comparison (C) experiments. (A) Embryos were static exposed to 0.1% DMSO between the 8–10 hpf start time and 58 hpf. Duration of burst activity (>5 pixels displaced during 1/30th s between consecutive frames) per minute for each individual ($n = 12$–16) was summed for 5 min in the dark following a 20 min acclimation period in the light. *Y*-axis depicts average duration of burst activity per minute. (B) Embryos were static exposed to BPA (0.001–10 µM) between the 8–10 hpf start time and 58 hpf, then duration of burst activity per minute for each individual ($n = 42$–47) was summed as above. Statistical significance was determined by one-way ANOVA, Student–Newman–Keuls post-test compared to vehicle-exposed embryos ($^{**}p < 0.01$). (C) Activity following exposure to 0.1 µM of the classical estrogen receptor agonist E2 or the ERRβ/γ agonist GSK4716 (GSK) compared to 0.1% DMSO control or 0.1 µM BPA ($n = 53$–57). Since this data did not pass a normality test (Shapiro–Wilk), significance was determined by one-way ANOVA on ranks (Kruskal–Wallis), Dunn's post-test, ($p < 0.05$).

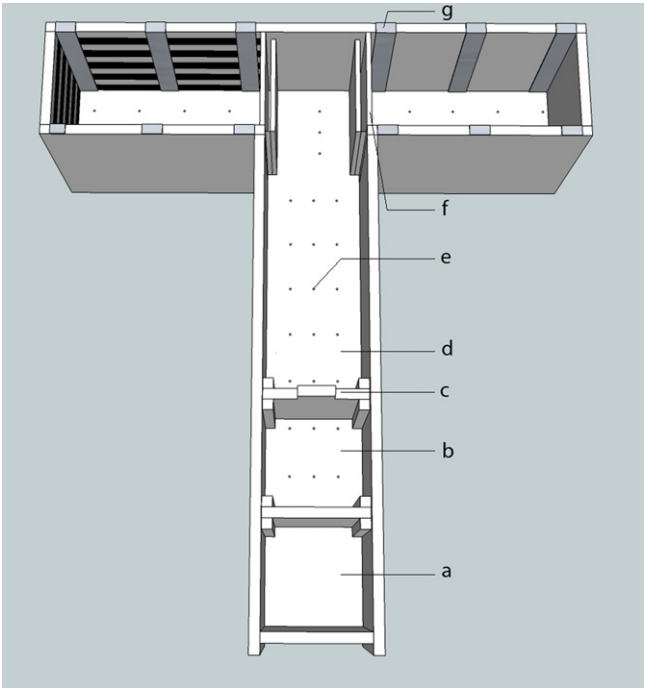

Fig. 4. T-maze used for adult learning task. a: drainage chamber; b: holding chamber; c: manually operated entry gate to raceway; d: raceway; e: perforated shim; f: computer operated gate to arm; g: electrode plate.

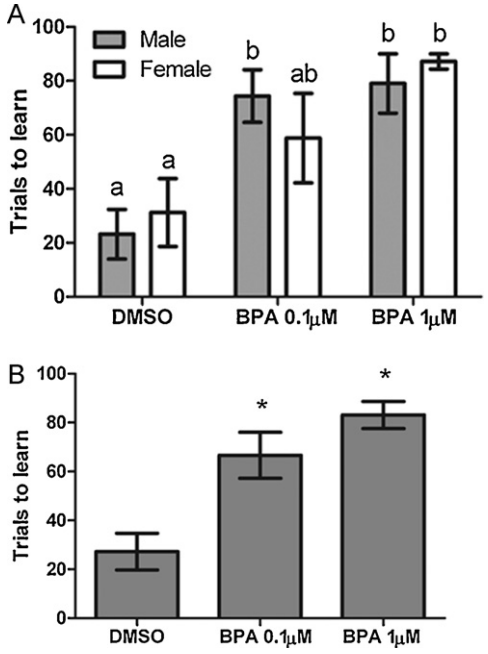

Fig. 5. Number of trials for adults to learn to select the correct arm in a T-maze following exposure to BPA during development (8–120 hpf) ($n = 5$). (A) Number of trials by sex to reach criterion in T-maze first reversal task. Zebrafish receive mild shock in first arm chosen in a T-maze. Criterion = 3 consecutive choices of opposite, non-shocking arm. Similar letters between exposure levels within same sex indicate no significant difference. Two-way ANOVA ($p < 0.001$). (B) Number of trials (sexes combined) to reach criterion in T-maze first reversal task. Zebrafish receive mild shock in first arm chosen in a T-maze. Criterion = 3 consecutive choices of opposite, non-shocking arm. One-way ANOVA ($^{*}p < 0.001$). Error bars depict SEM.

4. Discussion

Evidence is mounting in rodent model systems that developmental BPA exposure at human-relevant levels results in behavioral abnormalities and learning deficits (Jones et al., 2011; Nakamura et al., in press; Palanza et al., 2008). We demonstrated here that low-dose BPA exposure during CNS development caused larval hyperactivity and adult learning delays, establishing the zebrafish model as an additional resource for exploring the mode of action underlying BPA's neurobehavioral effects.

While other groups have utilized the embryonic zebrafish model to assess the developmental toxicity of both BPA and E2 (Duan et al., 2008; Fei et al., 2010; Gibert et al., 2011; Kishida et al., 2001; McCormick et al., 2010), these groups' assays differed from ours in exposure volume, duration, vehicle used, or endpoints assessed, leading to discrepancies among the study outcomes (Table 2) and highlighting a need to standardize testing methods. Quantifying stock concentrations is one way to compare results across research groups. Here, we derived a concentration–response curve using a BPA stock concentration that was clearly defined by HPLC analysis in order to most accurately link larval neurobehavioral endpoints to exposure concentration.

A second key component of our study was to measure BPA tissue dose associated with exposure concentrations using a novel application of HPLC heretofore not used in larval zebrafish studies. One major advantage of using this method is that it circumvented the need to use radio-labeled BPA standards. The main limitation of this assay was an inability to detect BPA in samples of 50 pooled embryos exposed to <1 μM BPA, necessitating extrapolation of tissue dose from exposure concentrations below 1 μM. Nevertheless, the uptake measurements reported here provide an important approximation in the embryonic zebrafish model associating waterborne exposure concentrations with tissue dose. Interestingly, the measured dose was less than the expected tissue concentration (e.g., 1 μM exposure concentration ≈228 μg/l ≈228 μg/kg (given the embryo is composed of >70% water (Hagedorn et al., 1997)); however, 1 μM exposure resulted in ~10 μg/kg tissue dose, ~20× less than 228 μg/kg). This suggests that the entire amount of BPA in the well was not absorbed by the embryo over the course of exposure and/or metabolism effectively reduced the measured tissue level.

Although unaccounted for factors such as absorption and metabolism make extrapolation between species a rough estimate, at best, it is important to note that the tissue doses associated with exposure concentrations ≤1 μM were in the range of human-relevant BPA levels (e.g., 1 μM waterborne exposure yielded roughly 10 μg/kg tissue dose, which is near the average level detected in human placenta (11.2 ng/g) (Schonfelder et al., 2002) and close to the maximum daily BPA intake of a bottle fed infant (11 μg/kg) (NTP, 2008)). Taken together, the larval behavior tests (roughly 8–58 hpf exposure) and the 8–58 hpf tissue dose approximation demonstrate that BPA doses ≤1 μg/kg measured shortly after peak CNS development (i.e., 16–36 hpf) were sufficient to produce significant hyperactivity in larval zebrafish. Thus, the concentrations to which fetuses and infants are potentially exposed (Braun et al., 2009; NTP, 2008; Schonfelder et al., 2002; Vandenberg et al., 2010) are associated with hyperactivity in this model.

BPA's developmental toxicity is commonly assumed to be mediated through ER agonism (e.g., Ben-Jonathan and Steinmetz, 1998). Thus, endogenous estrogen (17β-estradiol, E2) is an appropriate control for studies aimed at testing BPA's estrogen mimicry. Only one published study was found describing windows of exposure that elicit morphological defects following developmental E2 exposure in zebrafish (Kishida et al., 2001). Despite reported differences in E2 potency, the types of observed effects were consistent between the Kishida et al. (2001) and current studies (Table 2). The most noticeable consistency was a distinct curved body axis most apparent in our study in embryos exposed to 20 μM E2. Kishida and co-workers noted that this "curved tail down" phenotype has been associated with defects in CNS development, supporting the

Table 2
Comparison of bisphenol A (BPA) and 17β-estradiol (E2) toxicity studies conducted in the larval zebrafish model. *Note:* the caudal fin defect observed by Duan et al. (2008) was the only abnormality from among the other studies that was not observed in our study at some concentration.

Study	Kishida et al. (2001)		Duan et al. (2008)	McCormick et al. (2010)	Fei et al. (2010)	Gibert et al. (2011)	This study	
Chemical	BPA	E2	BPA	BPA	BPA	BPA	BPA	E2
Exposure protocol	Batch (40–50) exposure in 50 ml, daily solution renewal, DMSO vehicle, 2–72 hpf	Batch (40–50) exposure in 50 ml, daily solution renewal, DMSO vehicle, 2–72 hpf	Individual exposure in 2 ml, EtOH vehicle, 8–72 hpf	Batch exposure, 48 h	Batch (15) exposure in 25 ml, DMSO vehicle, 2–96 hpf	[a]Exposure protocol not clear, 5–48 hpf	Individual exposure in 100 μl, 8–120 hpf	Individual exposure in 100 μl, 8–120 hpf
LC_{50}	–	–	~73 μM (24 hpf)	–	–	–	>70 μM (120 hpf)	>25 μM (120 hpf)
NOAEC	–	–	9 μM (72 hpf)	–	–	–	30 μM (120 hpf)	0.1 μM (120 hpf)
Abnormalities at LOAEC	–	50% hatching delays at 10 μM	Edema, delayed hatching	15 μM (48 hpf) edema	30 μM (48 hpf) edema	>5 μM (48 hpf) otolith	>30 μM (120 hpf)	40% yolk sac edema at 10 μM
Mortality	20 μM, (statistical significance not reported)	80% at 10 μM	Significant at 24 hpf	Reported at 28 dpf for 5 (47%), 10 (22%), 15 (47%) μM	Observed at 30 μM at 48 hpf	Observed at 100 μM	None ~73 μM (120 hpf)	None 25 μM (120 hpf)
Notable abnormalities compared across studies	Curved tail down phenotype, but statistical significance not reported	Delayed hatch, pericardial edema, yolk sac edema, curved tail down	Pericardial edema and hatching delays, caudal fin defect	Pericardial edema	[b]Yolk sac and pericardial edema, uninflated swim bladder, delayed hatch	Otolith defects (50% of embryos at 25 μM)	Pericardial edema, delayed hatch, otolith defects >40 μM	Pericardial edema, yolk sac edema, curved tail down

hpf: hours post fertilization; dpf: days post fertilization; NOAEC: no observed adverse effect concentration; LOAEC: lowest observed adverse effect concentration; LC_{50}: concentration producing 50% mortality.
[a] Gibert et al. (2011) did not clearly state whether embryos were batch or individually exposed for the toxicity assay. Also, it is unclear whether the vehicle used was DMSO or ethanol (EtOH).
[b] Fei et al. (2010) reported abnormalities at 7 dpf.

idea that the CNS is the target organ system underlying behavioral abnormalities observed in E2-exposed embryos (Brand et al., 1996). Additionally, a recent study showed that E2 has a significant role in maintaining neuromuscular function and thus also impacts the peripheral nervous system (Houser et al., 2011). Although E2 is an appropriate control for all studies investigating the possibility that BPA causes neurobehavioral effects through estrogen mimicry, it is prudent to also include ligands of alternative receptors for which BPA exhibits high binding affinity.

As ERRγ binds BPA with substantially greater affinity than the classical ERs bind BPA (Okada et al., 2008; Washington et al., 2001), we included the synthetic ERRβ/γ agonist, GSK4716, as a second control for the early life-stage toxicity and larval behavior assays (Zuercher et al., 2005). This is the first study to describe the effects of GSK4716 exposure on developing vertebrates. The narrow margin of safety for GSK4716 (i.e., the mortality and abnormality concentration–response curves almost overlap) suggests that ERRβ/γ have significant roles in embryonic zebrafish development and exogenous activation of these receptors likely impacts multiple organ systems through pathways necessary for survival. The finding that ERRα, ERRβ, and ERRγ have significant roles in regulating energy metabolism (reviewed in Deblois and Giguere, 2011) supports the idea that ERRβ/γ activation by GSK4716 leads to mortality by interfering with metabolism in multiple organ systems. While the behavioral effects observed at non-lethal doses may also be the result of altered metabolism, it is probable that mortality/abnormality and hyperactivity are elicited through diverging underlying molecular events. It is also worth noting that the narrow margin of safety observed for GSK4716 could be associated with off-target effects (i.e., activation of receptors other than ERRβ or ERRγ.). Since few studies have investigated alternative GSK4716 targets and, to our knowledge, no study has investigated the targets of GSK4716 exposure at concentrations higher than 10 μM, further study is needed to explain this compound's toxicity. Although little is known about the activity of GSK4716 at doses higher than 10 μM, effects of GSK4716 $\leq$10 μM in skeletal muscle have been shown to be ERRγ-dependent through use of *in vitro* siRNA control experiments (Wang et al., 2010) demonstrating selectivity for its intended targets at the lower dose range.

This is the first study to examine the effects of low-dose BPA exposure on zebrafish behavioral endpoints. Preliminary testing determined that an exposure window between 10 and 58 hpf (48 h exposure) was sufficient to produce a locomotor effect in 5-day-old larvae (data not shown). This exposure window encompasses the primary wave of neurogenesis (16–36 hpf; (Kimmel et al., 1995)) and was used for all larval behavior tests (Note: an 8 hpf start time was found to yield identical results on behavior tests as using larvae first exposed at 10 hpf; the 8 hpf exposure was used for some trials to increase the number of embryos available for testing). We demonstrated here that exposure to 0.01 or 0.1 μM BPA during neurogenesis resulted in hyperactivity in the dark by 5 dpf zebrafish larvae. Exposure to higher BPA concentrations did not affect larval activity. Thus, the locomotor activity concentration–response curve was non-monotonic, consistent with the effects of BPA on *in vivo* reproductive endpoints (Weltje et al., 2005). The observation of an inverted U shape dose–response is consistent with that commonly observed for hormones such as E2 (reviewed in Kendig et al., 2010). Although this type of curve has been explained as an adaptation to a toxic response (e.g., the lack of response at higher concentrations is the result of adaptive detoxification mechanisms), a recent review on non-monotonic curves emphasizes that this is too simplistic of an explanation for what are certainly complex underlying events (Kendig et al., 2010). In the case of BPA, it has been proposed that such a curve is the result of the disruption of endogenous estrogen (Weltje et al., 2005).

Without further testing beyond the scope of this study, all that can be concluded by the observation of this concentration–response is that the mode of action of BPA is complex at the low concentration range (i.e., ≤10 μM) and does not follow a sigmoidal dose–response curve, which depicts the saturation of a target receptor with increasing dose. Therefore, this data is consistent with the involvement of multiple receptor targets and the idea that the receptor activity levels associated with the hyperactivity phenotype are not representative of saturation of available target receptors, whether one or multiple types. It is also important to note that this response was observed because we were specifically looking for the effects of BPA on behavior at low and more relevant exposure concentrations. If we had only considered morphological abnormalities, or had not included the concentrations between 0.001 and 1 μM, then this non-monotonic curve would not have been observed. We echo the warning by Kendig et al. (2010) that the potential to overlook subtle, non-monotonic responses is great and should be considered by researchers conducting low-dose BPA studies in zebrafish, particularly groups working from a definition of "low-dose" that may deem the lower concentrations tested herein irrelevant.

The evidence that BPA impacts neurodevelopment in the zebrafish model complements existing data from other model systems. While a range of behavioral effects have been reported in rodent models following prenatal BPA exposure (e.g., sex difference in mating behavior, anxiety, and learning and memory) (Negishi et al., 2004; Palanza et al., 2002)), it is nevertheless intriguing that several rodent studies have also shown increased locomotor activity following gestational or neonatal BPA exposure. For example, both Ishido et al. (2004) and Kiguchi et al. (2007) reported significant hyperactivity in rats several weeks following a single intracisternal dose of BPA delivered to 5-day-old pups. Additionally, Palanza et al. (2008) described greater activity by males in an open field test following prenatal low-dose (10 mg/kg) BPA exposure. It is important to note that zebrafish hyperactivity is a general swimming response that is not necessarily analogous to rodent or human hyperactivity. While it is possible this phenotype is representative of direct impact on CNS function, it could also be elicited through effects on neuromuscular functioning or impairment of sensory perception, any of which could be a manifestation of inappropriate ER or ERR activation.

To begin to test whether BPA's neurobehavioral effects are mediated through inappropriate ER or ERRγ activation, we included agonists of these receptors in the larval zebrafish behavior tests. Consistent with our findings for BPA, we found that exposure to 0.1 μM E2 or GSK4716 during neurogenesis resulted in hyperactivity in the dark by 5 dpf zebrafish larvae. While this is the first study in any vertebrate model system to investigate whether ERRγ activation by developmental GSK4716 exposure elicits any behavioral effects, estrogen exposure has previously been associated with increased activity in adult rodents (Morgan and Pfaff, 2002; Thomas et al., 1986) and has been used to rescue "immobility" in a rat forced swimming test, substantiating its role in maintaining normal activity levels (Estrada-Camarena et al., 2003). Although these studies dealt with adult exposures, they provide background supporting the importance of E2's effects on nervous system function. An additional study specifically addressed E2s effects on neurobehavioral development; Dugard et al. (2001) observed increased motor activity in offspring of rats exposed to 17α-ethinylestradiol during gestation. Although E2 has also been used to rescue the "listless" phenotype in embryonic zebrafish (Nelson et al., 2008), hyperactivity following low concentration E2 exposure has not been previously described in this model. In contrast, one study noted no effects of E2 on larval movement at lower doses, but significant loss of movement at a higher E2 dose (Hamad et al., 2007).

The hyperactivity following BPA exposure observed in our study is consistent with the increased activity observed in both rodents and larval zebrafish (this study) after E2 exposure. However, because GSK4716 exposure also elicited a hyperactive phenotype in our study, and GSK4716 is a selective ERRβ/γ agonist, this data is also consistent with an ERRγ agonism mode of action. More importantly, the similar phenotype following activation of different nuclear receptors demonstrates that zebrafish larval hyperactivity can be engendered through a variety of pathways. While ER and ERRγ activation is presumed to be central to the hyperactive phenotype elicited by E2 and GSK4716, respectively, many questions remain as to which organ systems are being affected and the identity of downstream gene targets. The hypothesis that larval hyperactivity is representative of a CNS effect is certainly bolstered by the adult data described here.

To investigate whether higher order brain functions such as learning are affected in adult fish by BPA exposure during critical periods of CNS development, we used a T-maze testing apparatus (Fig. 4) commonly used in both rodent and zebrafish studies (e.g., Grossman et al., 2010; Peitsaro et al., 2003). We showed here that exposure to the same concentration of BPA that caused significant hyperactivity in larval zebrafish (0.1 μM) led to significant deficits in learning a reversal task in adults 8 months after the initial BPA exposure. Similar learning deficits in spatial memory and avoidance tests following prenatal low-dose BPA exposure have been reported for mice (Negishi et al., 2004; Tian et al., 2010; Xu et al., 2010). Interestingly, no sex differences in behavior were detected in this study (further testing with a larger sample size will be required to confirm these findings). In contrast, there is a wealth of evidence for effects on sexually dimorphic behavior following prenatal BPA exposure (e.g., Jones et al., 2011; Negishi et al., 2004; Palanza et al., 2002). While the molecular events underlying BPA's effects on learning and memory are actively being investigated, with some evidence that neurotransmitter receptor expression may be altered (Ishido et al., 2004; Xu et al., 2010), it is generally postulated that inappropriate activation of ERs is central to BPA's mode of action (Ben-Jonathan and Steinmetz, 1998). The role of ERs in addition to other receptors of interest in eliciting BPA's effects on neurobehavioral development can be effectively investigated using the larval zebrafish model.

Here, we describe an early life-stage zebrafish assay that is ideal for investigating the mode of action by which BPA elicits long-term learning deficits, thus introducing a powerful tool to be used towards understanding BPA's neurobehavioral toxicity. Because transient BPA exposure during CNS development led to learning deficits in adults, we know that the events that permanently affected brain development and function were initiated during the early life-stage exposure. Therefore, although the persistence of neurobehavioral BPA toxicity is paramount to the relevance of this study, the power of this model lies in the identification of an early phenotype, larval hyperactivity, which resulted from exposure to the same concentrations that led to learning deficits in adults exposed during development. Although it is important to note that the larval and adult phenotypes are not necessarily representative of the same physiological events during the behavior tests, themselves (e.g., locomotion and learning are quite different behaviors), the larval phenotype can nevertheless be employed to further investigate the molecular events occurring during the developmental exposure that underlie BPA's neurobehavioral toxicity.

Taken together, the results of this study confirm the efficacy of using the zebrafish model to investigate the neurobehavioral effects of developmental BPA exposure. We have also demonstrated how the two compounds, E2 and GSK4716, can be used to investigate the roles of ERs and ERRγ, respectively, in eliciting the behavioral phenotypes described here.

Conflict of interest statement

None.

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