Bisphenol A exposure induces sensory processing deficits in larval zebrafish during neurodevelopment

Courtney Scaramella  
*California State University at Fullerton*  
Joseph B Alzagatiti  
*University of California, Santa Barbara*  
Christopher Creighton  
*California State University at Fullerton*  
Samandeep Mankatala  
*California State University at Fullerton*  
Fernando Licea  
*California State University at Fullerton*

See next page for additional authors

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Authors
Courtney Scaramella, Joseph B Alzagatiti, Christopher Creighton, Samandeep Mankatala, Fernando Licea, Gabriel M Winter, Jasmine Emtage, Joseph R Wisnieski, Luis Salazar, Anjum Hussain, Faith M Lee, Asma Mammootty, Niyaza Mammootty, Andrew Aldujaili, Kristine A Runnberg, Daniela Hernandez, Trevor Zimmerman-Thompson, Rikhil Makwana, Julien Rouvere, Zahra Tahmasebi, Gohar Zavradyan, Christopher S Campbell, Meghna Komaranchath, Javier Carmona, Jennifer Trevitt, David Glanzman, and Adam C Roberts

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Disorders of the Nervous System

Bisphenol A Exposure Induces Sensory Processing Deficits in Larval Zebrafish during Neurodevelopment

Courtney Scaramella,1,* Joseph B. Alzagatiti,2,* Christopher Creighton,1 Samandeep Mankatala,1 Fernando Licea,3 Gabriel M. Winter,1 Jasmine Emgate,3 Joseph R. Wisnieski,1 Luis Salazar,1 Anjum Hussain,4 Faith M. Lee,5 Asma Mammootty,6 Niyaza Mammootty,7 Andrew Aldujaili,8 Kristine A. Runnberg,1 Daniela Hernandez,1 Trevor Zimmerman-Thompson,4 Rikhil Makwana,9 Julien Rouvere,1 Zahra Tahmasebi,1 Gohar Zavradyan,4 Christopher S. Campbell,10 Meghna Komaranchath,11 Javier Carmona,12 Jennifer Trevitt,1 David Glanzman,8,13,14 and Adam C. Roberts1

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1Department of Psychology, California State University at Fullerton, Fullerton, CA 92831, 2Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, Santa Barbara, CA 93106, 3Department of Biology, California Institute of Technology, Pasadena, CA 91125, 4Department of Neuroscience, University of California, Riverside, Riverside, CA 92521, 5Department of Society and Genetics, University of California, Los Angeles, Los Angeles, CA 90095, 6Saint Louis University School of Medicine, St. Louis, MO 63104, 7Washington University School of Medicine, St. Louis, MO 63110, 8Department of Integrative Biology and Physiology, University of California, Los Angeles, Los Angeles, CA 90095, 9Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, 10Department of Neuroscience, The University of Arizona, Tucson, AZ 85719, 11Department of Biomedical Engineering, Columbia University, New York, NY 10027, 12Department of Physics, University of California, Los Angeles, Los Angeles, CA 90095, 13Department of Neurobiology, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA 90095, and 14Integrative Center for Learning and Memory, Brain Research Institute, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA 90095

Abstract

Because of their ex utero development, relatively simple nervous system, translucency, and availability of tools to investigate neural function, larval zebrafish are an exceptional model for understanding neurodevelopmental disorders and the consequences of environmental toxins. Furthermore, early in development, zebrafish larvae easily absorb chemicals from water, a significant advantage over methods required to expose developing organisms to chemical agents in utero. Bisphenol A (BPA) and BPA analogs are ubiquitous environmental toxins with known molecular consequences. All humans have measurable quantities of BPA in their bodies. Most concerning, the level of BPA exposure is correlated with neurodevelopmental difficulties in people. Given the importance of understanding the health-related effects of this common toxin, we have exploited the experimental advantages of the larval zebrafish model system to investigate the behavioral and anatomic effects of

Significance Statement

Bisphenol A (BPA) exposure elicits sensory processing deficits in larval zebrafish. Specifically, animals show abnormal prepulse inhibition (PPI) and short-term habituation (STH), which are behaviors mediated by a relatively simple neural circuit: the C-start escape circuit. Given the well-defined nature of the circuitry underlying the C-start reflex, the present study should facilitate future investigations of the neurobiological basis of BPA-induced behavioral deficits. Furthermore, the behavioral assays used here can be readily adapted for high-throughput screening of potential therapeutic agents. Finally, the present study provides a model system and a set of assays that can generally be used to investigate sensory processing, locomotion, anxiety, and key anatomic measurements.
BPA exposure. We discovered that BPA exposure early in development leads to deficits in the processing of sensory information, as indicated by BPA’s effects on prepulse inhibition (PPI) and short-term habituation (STH) of the C-start reflex. We observed no changes in locomotion, thigmotaxis, and repetitive behaviors (circling). Despite changes in sensory processing, we detected no regional or whole-brain volume changes. Our results show that early BPA exposure can induce sensory processing deficits, as revealed by alterations in simple behaviors that are mediated by a well-defined neural circuit.

Key words: autism spectrum disorder; C-start reflex; habituation; Mauthner cell; prepulse inhibition; zebrafish

Introduction
Bisphenol A (BPA) and BPA analogs are common chemicals found in polycarbonate plastics and other products. They are also used to help extend the shelf life of food products (Vandenberg et al., 2007; Chen et al., 2016). Because of the ubiquitous nature of BPA-containing products, measurable levels of BPA and BPA analogs are found in human breast milk, blood, and cerebral spinal fluid (Ye et al., 2006; Vandenberg et al., 2007; Stein et al., 2015). Fetal plasma and amniotic fluid contain detectable levels of BPA, indicating that it can cross the maternal-fetal-placental barrier and influence fetal development (Schonfelder et al., 2002; Yamada et al., 2002). Thus, BPA can potentially impact human development in utero and postnatally.

BPA exposure is associated with human disease and dysfunction, including fertility problems, cancer, obesity, and neurobehavioral abnormalities (Rochester, 2013). In particular, BPA exposure is correlated with the development of neurodevelopmental disorders, such as autism spectrum disorder (ASD), and children diagnosed with ASD have higher BPA metabolites in their urine (de Cock et al., 2012; Stein et al., 2015; Yoo et al., 2020; Hansen et al., 2021). Further, multiple studies have shown that behavioral problems correlate with maternal BPA exposure (Ejaredar et al., 2017).

BPA can potentially disrupt cellular function through multiple molecular mechanisms, including disruption of endocrine function and chromosomal modifications. BPA perturbs endocrine function and can bind to several endocrine-related receptors, such as estrogen receptors (Mustieles et al., 2015). BPA exposure changes gene expression by modulating transcription factors and perturbing the epigenome (Dolinoy et al., 2007; Yaoi et al., 2008; Huang et al., 2021; Scopel et al., 2021; Sundarraj et al., 2019). Finally, BPA disrupts double-stranded DNA repair mechanisms and increases oxidative stress (Isso et al., 2006; Allard and Colaíáçovo, 2010; Durovcova et al., 2018).

Rodents have been used extensively to model human neurodevelopmental disorders, such as ASD. The molecular perturbations underlying rodent models of ASD result in social deficits, repetitive behaviors, and cognitive inflexibility (Moy et al., 2006; Ellegood and Crawley, 2015; Kazdoba et al., 2016). In mice, prenatal BPA exposure elicits several ASD-like phenotypes, including increased anxiety, repetitive behaviors, sensory processing difficulties, and impairments in memory and social behaviors. The impairments observed in mice exposed to BPA suggest that this toxin might contribute to human ASD or related neurodevelopmental difficulties (Wolstenholme et al., 2013; Hu et al., 2022).

Modeling human BPA exposure in animals is difficult because the half-life of BPA is ~6 h, and these measurements only represent a snapshot of exposure (Thayer et al., 2015). Human samples do not provide information regarding the range of BPA exposure or the impact of exposure to the toxin at critical periods of human development. Understanding the developmental consequences of BPA exposure, which is known to modify DNA methylation, is particularly important because a single application of a DNA methyltransferase inhibitor in mice at postnatal day (P)7 induces life-long behavioral abnormalities and deficits in synaptic plasticity (Subbanna et al., 2016).

Unlike the complications involved in exposing common laboratory mammals to toxins because of their in utero development, chemical agents can be efficiently and effectively delivered to embryonic and larval zebrafish ex utero. It has been found that exposure to relatively high concentrations of BPA early in the zebrafish life cycle increases mortality and deformity, and delays hatching. Interestingly, despite general agreement regarding the adverse effects of high levels of BPA on mortality, body shape, and time of hatching, the reported threshold concentration of BPA for producing these effects varies greatly (Lam et al., 2011; Saili et al., 2012; Tse et al., 2013; Wang et al., 2013; Martinez et al., 2018; Olsvik et al., 2019; Coumailleau et al., 2020; Gyimah et al., 2021; Huang et al., 2021; Scopel et al., 2021; Sundarraj et al., 2021).
Furthermore, some studies have found that BPA exposure modifies locomotion in zebrafish, whereas others have not (Saili et al., 2012; Wang et al., 2013; Kinch et al., 2015; Fraser et al., 2017; Olsvik et al., 2019; Coumalleau et al., 2020; Gyimah et al., 2021; Wu et al., 2021). Given the complexity of the underlying mechanisms of behavior, it is not surprising that BPA exposure can produce widely disparate behavioral results. Nonetheless, exposing larval zebrafish to BPA clearly modifies neural circuitry involved in locomotion and, therefore, BPA exposure during development would be expected to cause behavioral alterations. Here, we investigated the consequences of early BPA exposure on sensory processing, locomotion, and anxiety in zebrafish larvae, as well as on anatomy.

Materials and Methods

Animals

The TL strain of zebrafish was used for all experiments except for those in which brain volume was measured. For the measurements of brain volume, we used transgenic zebrafish that express the photoconvertible protein Kaede pan-neuronally Tg(elavl3:Kaede)RW0130a (RRID: ZFIN ID: ZDB-GENO-060619-2; Sato et al., 2006).

BPA exposure

After zebrafish eggs were collected, they were placed in E3 water (5 mM NaCl, 0.33 mM MgSO4, 0.33 mM CaCl2, 0.17 mM KCl, 1 mM HEPES, 10^{-5}M methylene blue, pH 7.0) and transferred to the laboratory for staging. Embryos were staged to ~4–5 h after fertilization; they were then placed in Petri dishes containing E3 water alone, E3 water containing 0.05% dimethyl sulfoxide (DMSO), or E3 water containing BPA (10–50 μM) in 0.05% DMSO, which were put into an incubator (14/10 h light/dark; 28.5°C). Each day, the dishes were cleaned by replacing the solutions in the Petri dishes, and any dead embryos were removed and counted. On the fifth day of exposure, all solutions were replaced with E3, and the larvae were maintained in E3 until testing at 6 d post-fertilization (dpf). On testing day, fish were fed premixed food (Hatchfry Encapsulon) in E3. This food was removed 4 h later, the solution in the Petri dishes was replaced with fresh E3, and then experimental protocols were begun (≥1 h after the removal of the food).

Analyses of survival and deformity

In experiments designed to determine minimum BPA concentrations that caused death, deformities, or hatching delays, we assayed the effect of varying concentrations of BPA on these variables. By the end of the sixth day, the total number of fish that survived was calculated for each plate of embryos to determine mortality (0 = alive at 6 dpf, 1 = dead at 6 dpf). Concurrently, deformities of the surviving embryos were recorded (0 = no deformity at 6 dpf, 1 = deformity at 6 dpf) and photographed. Common deformities included spinal/tail bends, cranial malformations, and yolk and pericardial edema. Failure to escape the chorion by 6 dpf was also classified as a deformity. Animals with deformities were euthanized on 6 dpf and were not used for behavioral experiments. Finally, to determine whether hatching was delayed (Kimmel et al., 1995), the day the embryos escaped from their chorions was recorded (1 = hatched after 3 dpf or failed to hatch, 0 = hatched before 3 dpf).

Behavioral protocols

Audiogenic startle

To measure the C-start reflex, surviving and nondeformed zebrafish larvae (6 dpf) were placed into individual wells with a 1.63-cm diameter (~3 ml of E3) in a 24-well plate. The plate was then positioned on a lightbox (Gagne Inc.) next to a speaker. The volume of the speaker was calibrated in air with a decibel meter (re 20 μPa). After a 30-min period of acclimation, six auditory/vibrational (AV) pulses (200 Hz, 108 dB re 20 μPa, 2-ms pulse duration) were delivered every 5 min. A high-speed camera (Mako U-029B; Allied Vision) positioned above the plate was used to record (500 fr/s) the responses of the larvae to the AV pulse. The determination of whether or not an AV pulse elicited a C-start was made by visual inspection of the video recording by a blind observer. Long-latency startle responses (>26 ms) were not counted as C-starts; such long-latency startles are mediated by different neurocircuitry than are the shorter latency C-starts (Liu and Fetcho, 1999; Burgess and Granato, 2007; Kohashi and Oda, 2008).

Prepulse inhibition (PPI)

Zebrafish larvae (6 dpf) were placed into individual wells (see above) and allowed to acclimate for 30 min. After this acclimation period, six AV stimuli were delivered (5-min intertrial interval), and C-start reflexes were recorded and quantified as described above. AV stimuli were alternatively delivered with or without a prepulse auditory stimulus. The prepulse stimulus was 100 ms in duration (200 Hz, 55 dB re 20 μPa) and preceded the startle stimulus (2-ms duration, 500 Hz, 108 dB re 20 μPa) by 100 ms. The relative difference in startles with and without the prepulse stimulus was used to calculate the PPI index [(percentage startle response without the prepulse stimulus – percent startle response with the prepulse stimulus)/(the average startle response without a prepulse)].

Short-term habituation (STH)

To measure STH, larvae (6 dpf) were placed into individual wells in a 24-well plate as described above. After 30 min of acclimation, 120 AV pulses (200 Hz, 2-ms duration, 108 dB) were delivered at 1 Hz, followed by a posttest AV stimulus 30 s after the last training stimulus. The responses to the training and posttest pulses were video recorded, and only short-latency (C-start) responses were counted for the determination of habituation.

Thigmotaxis

Twenty zebrafish larvae (6 dpf) were put into a Petri dish (142 mm in diameter) containing ~100-ml E3; the dish was placed inside a custom-made testing chamber with overhead illumination. The larvae were given 30 min to
acclimate, after which a camera (Casio Exilim EX-ZR1000, EX-ZR800, or EX-ZR100; Casio America) was used to record a single image of the positions of all 20 fish in relation to the center and edge of the Petri dish. Using ImageJ (RRID:SCR_003070; Schneider et al., 2012), an average plate position for the fish was determined from the image; for statistical analyses, each plate average equaled an \( n \) of 1.

**Locomotion**

Zebrafish larvae (6 dpf) were placed individually into a Petri dish (36 mm in diameter) containing 8-ml E3. After 1 min, the movement of the fish was recorded for 20 min (50 f/s) by a camera (Casio Exilim EX-ZR1000, EX-ZR800, or EX-ZR100; Casio America) positioned above the Petri dish. The position of each fish during the 20-min observation period was sampled every 41.67 ms and the total distance (cm) the fish moved was calculated using Behavioral Cloud software. Also, the path taken by each fish was determined by sampling the fish’s position every 1 s. Using ImageJ, a blind observer then counted the total number of circles made by the fish during the 20-min period; this number was used as a measure of repetitive behavior (circling). Movements were considered to constitute a circle if the path taken by a fish followed a circular trajectory. A circle-like movement was only counted if the path was 75% complete (closed) before a change in direction. Additionally, circles that included more than half of the dish were not included in the circle count because they were difficult to distinguish from mere swimming in a circular enclosure (the dish).

**Anatomical measurements**

**Head size**

Zebrafish (6 dpf) were put into tricaine (MS-222; 200 mg/l) and then positioned dorsal side up in 1% low melting point agarose. The fish were then imaged using an AmScope stereo microscope and photographed with an attached digital camera. ImageJ was used to determine the area of the dorsal portion of the head in the photographs by a blind observer. The head was outlined, starting posterior to the eyes, as indicated in Figure 1.

**Table 1: Statistical analyses**

| Data structure | Type of test | Power (\( \alpha = 0.05 \)) |
|----------------|-------------|-----------------------------|
| a(Fig. 2A)     | One-way ANOVA test | 0.92                        |
| b(Fig. 2B)     | One-way ANOVA test | 1.00                        |
| c(Fig. 2C)     | One-way ANOVA test | 1.00                        |
| d(Fig. 2D)     | One-way ANOVA test | 0.09                        |
| e(Fig. 3A)     | Unpaired t test  | 0.07                        |
| f(Fig. 3B)     | Unpaired t test  | 0.19                        |
| g(Fig. 3C)     | Unpaired t test  | 0.30                        |
| h(Fig. 4A)     | Unpaired t test  | 0.17                        |
| i(Fig. 4B)     | Unpaired t test  | 0.63                        |
| j(Fig. 4C)     | Unpaired t test  | 0.07                        |
| k(Fig. 5)      | Unpaired t test  | 0.63                        |
| l(Fig. 6A)     | Two-way ANOVA test (interaction) | 1.00                        |
| m(Fig. 6B)     | One-way ANOVA test | 1.00                        |
| n(Fig. 6C)     | One-way ANOVA test | 0.79                        |
| o(Fig. 7)      | Unpaired t test  | 0.12                        |
| p(Fig. 8A)     | Unpaired t test  | 0.06                        |
| q(Fig. 8B)     | Unpaired t test  | 0.30                        |
| r(Fig. 9)      | Unpaired t test  | 0.36                        |
| s(Fig. 10B)    | Two-way ANOVA test (interaction) | 0.05                        |
| t(Fig. 10B)    | Two-way ANOVA test (main effect) | 0.06                        |
| u(Fig. 10C)    | Unpaired t test  | 0.06                        |

Figure 1. Image of a zebrafish larva illustrating the area used for head size measurements. Dorsal view of a zebrafish larvae embedded in low melting point agarose. Red lines outline the area measured to determine the head size of larvae. Scale bar: 150 μm.
Brain size
Images of zebrafish (6 dpf) expressing the photoconvertible protein Kaede pan-neuronally driven by the ELAV3 promoter (Sato et al., 2006) were used to determine the whole-brain volume and volumes of brain regions in zebrafish exposed to BPA. To image zebrafish brains, an LSM Pascal microscope (Zeiss) was used (488-nm excitation) to construct a whole-brain image stack using z-stacks (2-μm slices) and tiling. The tiles were stitched together after the images were taken. In addition, we used depth-dependent adaptive illumination to ensure adequate detection of deeper brain regions. The calculated volumes from the 2-μm slices were then summed to determine the total volume for each region (forebrain, midbrain, and hindbrain), and these values were then added to quantify the total brain volume.

Pharmacology
All compounds were obtained from Sigma-Aldrich. BPA (CAS 80-05-7) was dissolved in DMSO (CAS 67-68-5) to make a final concentration of 0.05%.

Statistical analyses
Group comparisons were determined by unpaired t tests or ANOVAs. When appropriate, Tukey’s HSD tests were used for post hoc analyses. Table 1 includes a summary of the statistical analyses.
Extended exposure to DMSO does not increase mortality, delays in hatching, or abnormal sensorimotor responses. A. Exposure of zebrafish larvae to DMSO (0.05%) from 0 to 5 dpf did not change the mortality rate (DMSO group = 22.00 ± 5.92%, n = 50) compared with a control group exposed only to E3 (E3 group = 18.75 ± 4.39%, n = 80) according to an unpaired t test (t_{128} = 0.45, p = 0.66). B. Extended exposure (5 d) to DMSO (0.05%) did not significantly delay hatching in zebrafish compared with fish exposed to E3 solution (DMSO group = 2.50 ± 2.50%, n = 40; E3 group = 5.8 ± 3.28%, n = 66; unpaired t test; t_{66} = 1.09, p = 0.28). C. An unpaired t test (t_{104} = 1.45, p = 0.15) revealed no significant differences in startle response probability between the DMSO group (88.33 ± 3.00%, n = 40) and the E3 group (93.18 ± 8.77%, n = 66).

Results

Exposure to high levels of BPA increases mortality and deformity in larval zebrafish

To determine the highest dose of BPA exposure that does not elicit increased mortality and deformity, or reduced sensorimotor responsiveness, we exposed zebrafish larvae to several doses of BPA for 5 d (Fig. 2). DMSO (0.05%) was used as the vehicle control and was included in the control group’s solutions for all experiments investigating BPA exposure. Extended exposure (5 d) to E3 or DMSO (0.05%) alone did not significantly alter the rate of larval mortality (Fig. 3A) or deformity (data not shown); no deformities were observed for either the E3 group (0/66) or DMSO group (0/40). Furthermore, there were no hatching delays (Fig. 3B) or reductions in sensorimotor responsiveness, as measured by startle to an auditory stimulus (Fig. 3C), in either the E3 or DMSO groups. High concentrations (50 μM) of BPA, however, increased mortality (Fig. 2A; Table 2). There was also an increased likelihood of deformity with levels of BPA ≥30 μM (Fig. 2B; Table 2). Relatively low concentrations (10 μM) of BPA significantly delayed hatching, as did higher concentrations (>30 μM; Fig. 2C; Table 2), this effect might indicate a neurodevelopmental delay or changes to the chorion that make escaping the chorion more difficult. No significant reductions in audiogenic startle rate were observed with BPA concentrations ≤30 μM (Fig. 2D; Table 2). Animals with visually detectable deformities were not used to test for startle responses. Consequently, no zebrafish larvae exposed to concentrations of BPA >40 μM were tested for startle.

A recent report by Scopel et al. (2021) investigated the effect of 25 μM BPA on mortality, deformity, and hatching in larval zebrafish. Because Scopel et al. (2021) only exposed zebrafish to 25 μM BPA for 3 d, we investigated the consequences of a 5-d exposure of larvae to 25 μM BPA. As shown in Figure 4A, larvae exposed to 25 μM BPA did not exhibit a statistically significant increase in mortality (BPA group = 3.33 ± 3.33) compared with the vehicle control group (DMSO group = 0.00 ± 0.00). Furthermore, no deformities were observed in either the BPA group (n = 29) or the DMSO control group (n = 30; data not shown). We did detect (Fig. 4B) a significant delay in hatching in the BPA group (43.33 ± 9.20) compared with the DMSO group (16.67 ± 6.92). With respect to the percent evoked startle response, there were no significant differences observed between the BPA group (92.53 ± 2.12) and the DMSO group (93.89 ± 2.19; Fig. 4C). Based on these findings, 25 μM was selected as the experimental concentration to investigate the behavioral and anatomic effects of BPA exposure from 0 to 5 dpf.

Table 2: Mortality, deformity, hatching delays, startle response in zebrafish larvae exposed to BPA

| BPA  | Mortality: mean and SEM | Deformity: mean and SEM | Hatching delays: mean and SEM | Startle response: mean and SEM |
|------|-------------------------|-------------------------|------------------------------|-------------------------------|
| 0 μM | 16.67 ± 6.92%           | 0.00 ± 0.00%            | 4.00 ± 4.00%                 | 88.67 ± 4.38%                |
|      | n = 30                  | n = 25                  | n = 25                       | n = 25                       |
| 10 μM| 13.33 ± 6.31%           | 11.54 ± 6.39%           | 44.83 ± 9.40%                | 91.30 ± 3.61%                |
|      | n = 30                  | n = 26                  | n = 29                       | n = 23                       |
| 20 μM| 20.00 ± 7.43%           | 8.33 ± 5.76%            | 28.00 ± 9.17%                | 93.18 ± 4.74%                |
|      | n = 30                  | n = 24                  | n = 25                       | n = 22                       |
| 30 μM| 33.33 ± 8.75%           | 30.00 ± 10.51%          | 55.00 ± 11.41%               | 92.86 ± 4.85%                |
|      | n = 30                  | n = 20                  | n = 20                       | n = 14                       |
| 40 μM| 23.33 ± 7.85%           | 100.00 ± 0.00%          | 56.52 ± 10.57%               | n/a                           |
|      | n = 30                  | n = 23                  | n = 23                       |                              |
| 50 μM| 53.33 ± 9.26%           | 100.00 ± 0.00%          | 95.00 ± 5.00%                | n/a                           |
|      | n = 30                  | n = 14                  | n = 20                       |                              |
BPA exposure disrupts sensorimotor gating in zebrafish larvae

As measured by PPI, deficits in sensorimotor gating have been observed in humans with neurodevelopmental disorders, such as schizophrenia and ASD (Braff et al., 1978, 2001; Grillon et al., 1992; McAlonan et al., 2002; Frankland et al., 2004; Perry et al., 2007; Kohl et al., 2013; but see Ornitz et al., 1993; Madsen et al., 2014). Further, animal models of these developmental disorders also display deficits in PPI (Dulawa et al., 1997; Swerdlow and Geyer, 1998; Burgess and Granato, 2007; Bickel et al., 2008; Möhrle et al., 2021). To determine whether BPA exposure causes deficits in PPI, we treated zebrafish with 25 μM BPA from 0 to 5 dpf. At 6 dpf, we gave BPA-treated (experimental) and DMSO-treated (control) larvae brief auditory stimuli to elicit a C-start, either with or without a prepulse stimulus. We calculated the level of PPI for experimental and control zebrafish. As shown in Figure 5, prolonged exposure to 25 μM BPA significantly reduced PPI (BPA group = 34.74 ± 4.03) compared with vehicle-treated controls (DMSO group = 47.99 ± 4.08). Thus, BPA exposure appears to affect the neural circuitry mediating sensorimotor gating.

BPA exposure disrupts STH in zebrafish larvae

Neurodevelopmental disorders exhibit cognitive difficulties, including deficits in habituation, suggesting problems processing sensory information (Barry and James, 1988; Bolino et al., 1992, 1994; Braff et al., 1992; Martineau et al., 1992; Vivanti et al., 2018; Bharath et al., 2020; Gandhi et al., 2021; Jamal et al., 2021). Similar to humans with neurodevelopmental disorders, mouse models of neurodevelopmental disorders also exhibit reductions in habituation (Dulawa et al., 1997; Restivo et al., 2005; Bickel et al., 2008; Lovelace et al., 2016; Möhrle et al., 2021). The extensive literature on habituation in zebrafish larvae (Eaton et al., 1977; Roberts et al., 2011, 2013, 2016, 2019; Wolman et al., 2011) makes this model organism particularly well suited for investigating abnormal habituation resulting from neurodevelopmental disorders. To test the effect of chronic exposure to BPA on STH in zebrafish larvae, we gave larvae 120 AV pulses (1 Hz). There was significantly less habituation (more startle responses) during training in BPA-treated larvae (BPA group = 10.25 ± 0.98 startles) than in the control group (DMSO group = 3.45 ± 0.11; Fig. 6A, B). The BPA-treated group also showed significantly less habituation (higher probability of startle) on a posttest taken 30 s after the end of habituation training (BPA group = 0.40 ± 0.11) than did the control group (DMSO group = 0.05 ± 0.05; Fig. 6C).

Thigmotaxis was not altered by exposure to BPA

Organisms tend to move toward the edges of an open area when anxious (thigmotaxis), and their position in space can serve as a measurement of anxiety (Christmas and Maxwell, 1970; Prut and Belzung, 2003; Schnörr et al., 2012; Ahmad and Richardson, 2013; Walz et al., 2016). To assess whether 5 d of exposure to BPA affects...
thigmotaxis in zebrafish, we quantified their propensity to thigmotax when put into a Petri dish (142 mm in diameter). As shown in Figure 7, we did not observe a significant difference between BPA-treated zebrafish (BPA group = 17.27 ± 0.69 mm) and control zebrafish (DMSO group = 16.40 ± 0.89 mm) with respect to their distance from the dish’s edge. Thus, BPA exposure did not increase anxiety in zebrafish, at least as measured by this assay.

BPA exposure did not modify locomotion in zebrafish larvae

In contrast to the results of Saili et al. (2012), we did not find that BPA exposure increased locomotion. The BPA group traveled 418.86 ± 22.33 cm during the 20-min test period, whereas the control (DMSO) group traveled 407.33 ± 30.62 cm (Fig. 8A). We also tested whether BPA exposure increased the propensity for larvae to perform repetitive behaviors (Dwivedi et al., 2019). For this test, we quantified the number of circles made by zebrafish larvae during the same 20-min test period (Fig. 8B). The number of circles made during swimming by the BPA-treated group (19.41 ± 2.22) was not different from the number made by the DMSO-treated group (23.90 ± 2.18; Fig. 8B). Thus, BPA exposure did not produce any significant changes in this repetitive behavior.

Head size and brain volume were unchanged in zebrafish larvae by BPA exposure

We wished to know whether BPA exposure affects head size in zebrafish larvae. Accordingly, we measured
the area of the dorsal portion of the head, as indicated in Figure 1. We found no difference in head size between a group of zebrafish exposed to BPA for 5 d (BPA group = 12,792,608.93 m$^3$) and a control group (DMSO group = 86,742.87 m$^3$; Fig. 9). Thus, our BPA treatment did not affect larval head size.

We also examined whether brain volume was altered in larvae exposed to BPA. Therefore, we measured the volume of the forebrain, midbrain, and hindbrain in fish treated with BPA or DMSO alone. We observed no significant differences in the volume of the forebrain (BPA group = 12,792,608.93 m$^3$; DMSO group = 13,046,271.33 m$^3$), midbrain (BPA group = 9,472,418.03 m$^3$; DMSO group = 9,186,811.83 m$^3$), and hindbrain (BPA group = 15,168,013.01 m$^3$; DMSO group = 15,168,202.24 m$^3$; Fig. 10A). Additionally, we summed these brain area volumes to determine whole-brain volume in the larvae. The whole-brain volume did not differ significantly in the BPA-treated (37,433,229.20 m$^3$) and the vehicle-treated (DMSO = 37,832,896.17 m$^3$) groups (Fig. 10B).

Discussion

Here, we examined the effect of early exposure to BPA, an environmental toxin, on development in the zebrafish. To date, most investigations of the behavioral ramifications of BPA exposure have focused on locomotion; there has been little emphasis on sensory processing despite evidence that humans and animal models of neurodevelopmental disorders commonly involve disruptions of sensory processing, including PPI and habituation (Braff et al., 1978, 1992; Barry and James, 1988; Bolino et al., 1992, 1994; Grillon et al., 1992; Martineau et al., 1992; Dulawa et al., 1997; Swerdlow and Geyer, 1998; Braff et al., 2001; McAlonan et al., 2002; Frankland et al., 2004; Restivo et al., 2005; Burgess and Granato, 2007; Perry et al., 2007; Bickel et al., 2008; Kohl et al., 2013; Lovelace et al., 2016; Vivanti et al., 2018; Bharath et al., 2020; Gandhi et al., 2021; Jamal et al., 2021; Möhrle et al., 2021; but see Ornitz et al., 1993; Madsen et al., 2014). Both PPI and habituation test the ability of an organism to ignore unnecessary sensory information. We found reductions in PPI and habituation (during and 30 s after training) of the C-start reflex in larvae exposed to BPA (Figs. 5, 6). Because the neural circuitry and molecules underlying these two behavioral phenomena are well-described in larval zebrafish (Liu and Fetcho, 1999; Burgess and Granato, 2007; Kohashi and Oda, 2008; Roberts et al., 2011, 2019; Wolman et al., 2011; Kohashi et al., 2012; Bergeron et al., 2015; Marsden and Granato, 2015; Wolman et al., 2015; Tabor et al., 2018; Nelson et al., 2020; Zooodsma et al., 2020), elucidation of the BPA-induced pathology should be relatively straightforward. Furthermore, habituation and PPI of the C-start reflex are behaviors that are well-suited for high-throughput drug screening, which should facilitate the development of novel treatments to mitigate the effects of BPA (Rihel et al., 2010; Thyme et al., 2019).

We found that 25 μM BPA was the highest level of exposure (5 d) that did not result in an increase in gross deformities and significant mortality (Figs. 2, 4). Generally, this finding is consistent with the literature; however, the exact level of BPA to elicit these effects varies greatly (Lam et al., 2011; Saill et al., 2012; Tse et al., 2013; Wang et al., 2013; Martinez et al., 2018; Olsvik et al., 2019; Coumailloux et al., 2020; Gyimah et al., 2021; Huang et al., 2021; Scopel et al., 2021; Sundarraj et al., 2021; Wu et al., 2021). The differences in the reported concentrations of BPA required to elicit deformity and mortality likely result from variation in experimental protocols or the strain of zebrafish used. Similar to deformity and mortality, hatching delays have more commonly been reported with higher levels of BPA (Martinez et al., 2018; Scopel et al., 2021; but see Olsvik et al., 2019 compared with lower levels of BPA (Gyimah et al., 2021; Huang et al., 2021; Gu et al., 2022; but see Sundarraj et al., 2021). By contrast, very

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**Figure 8.** BPA exposure did not modify locomotion in zebrafish larvae. A. Mean distance traveled by BPA-exposed ($n = 32$) and DMSO-exposed ($n = 31$) fish (unpaired $t$ test; $t_{61} = 0.30$, $p = 0.77$). B. Number of circles made by larvae exposed to BPA (BPA group, $n = 32$) and the vehicle (DMSO group, $n = 31$; $t_{61} = 1.44$, $p = 0.15$).

**Figure 9.** Exposure to BPA did not change head size in zebrafish larvae. Mean volume of the head in BPA-treated (BPA group, $n = 30$) and vehicle-treated (DMSO group, $n = 30$) fish ($t_{60} = 1.63$, $p = 0.11$).
low levels of BPA have been reported to accelerate hatching (Qiu et al., 2016; Coumailleau et al., 2020). We observed hatching delays at BPA concentrations as low as 10 μM, as well as at higher concentrations (Figs. 2C, 4B). Changes to the chorion might be responsible for the observed delays; however, we think it is more likely that hatching latencies stem from delays in neural or skeletomuscular development. Notice that early BPA exposure in our study did not diminish the probability of startle (C-start reflex), which suggests that the BPA-treated zebrafish were not in poor health at the time of testing (Figs. 2D, 4C).

In humans, neurodevelopmental disorders such as ASD are often comorbid with anxiety (Lai et al., 2019), animal models of ASD also tend to exhibit comorbidity with anxiety, as indicated in such tasks as the open field test in mice (Hung et al., 2008; Blundell et al., 2009; Schmeisser et al., 2012; Clipperton-Allen and Page, 2014). In 5-dpf zebrafish larvae, Fraser et al. (2017) found that BPA exposure (10 μM) increased anxiety, as measured by thigmotaxis. By contrast, we did not detect a change in thigmotaxis despite using a higher concentration of BPA (25 μM; Fig. 7). While several experimental parameters (see below) differed between our experiments and those of Fraser et al. (2017), they measured the effect of BPA exposure while the toxin was present at the time of testing, whereas BPA was not present during behavioral testing in our experiments. Therefore, the enhanced thigmotaxis observed by Fraser et al., may be an acute effect of BPA. In addition, we used a larger behavioral chamber than did Fraser et al. (2017). Furthermore, our measure of thigmotaxis was the position of the zebrafish as a continuous variable (distance from the edge; Roberts et al., 2020) rather than the level of activity within a defined zone in the chamber (Schnörr et al., 2012). The larger chamber combined with a continuous variable should have increased the likelihood of detecting baseline thigmotaxis compared with alternative methods. As such, we think the different concentrations of BPA or exposure models most likely account for the different results. Consequently, we believe that the differences between our results and those of previous studies of BPA’s effects on thigmotaxis in zebrafish can be ascribed to differences in experimental variables, including differences in the concentrations of BPA used.

Numerous studies have found that BPA affects locomotion in zebrafish. However, there is little consistency in reported behavioral outcomes (Saili et al., 2012; Wang et
al., 2013; Kinch et al., 2015; Fraser et al., 2017; Olsvik et al., 2019; Coumailleau et al., 2020; Gymah et al., 2021; Wu et al., 2021). We observed no changes in the total distance moved or in repetitive behavior (circling; Fig. 8) in BPA-exposed fish. Possibly, the disparities in results of the studies on locomotion can be attributed to methodological differences. Fraser et al. (2017) observed that the presence or absence of a light cycle and the size of the testing chamber could substantially influence BPA’s effect on locomotion. Fraser and colleagues also claimed that BPA-induced hyperlocomotion requires dark rearing, and our larvae were reared with a day/night cycle.

BPA has been reported to modify neural structure and function in mice (Inadera, 2015) and to increase neurogenesis in the hypothalamus of zebrafish (Kinch et al., 2015). Studies of BPA’s effect on neurogenesis throughout the central nervous system have yielded conflicting reports (Gymah et al., 2021; Gu et al., 2022). We found no BPA-related change in the volume of the forebrain, midbrain, or hindbrain, or in total brain volume (Fig. 10). This suggests that the PPI and habituation deficits could not have resulted from any gross changes in brain morphology. However, our results do not preclude the possibility of more subtle BPA-induced modifications in neural morphology.

Model developmental systems such as larval zebrafish, which permit in-depth analyses of toxin-induced molecular, cellular, and behavioral changes, are important for understanding how chronic exposure to environmental toxins such as BPA disrupts neural development and behavior. The behavioral assays used in our study revealed the disruptive effects of BPA exposure on sensory processing and learning in zebrafish larvae. Our finding that both PPI and STH of the C-start are abnormal in BPA-exposed larvae should facilitate the identification of the neurobiological causes of the perturbations of sensory processing caused by early BPA exposure.

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