Supporting Information

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SI Materials and Methods

Zebrafish Husbandry and Contaminant Preparation. Wild-type TL (Tail Long) zebrafish embryos were maintained on a 14-h light:10-h dark cycle at 28 °C in embryo medium (E3) as described by Westerfield, et al. (1). All contaminant treatments were prepared in a 1:3 ratio of 0.002% (vol/vol) 1 M NaOH to 95% EtOH (vehicle) in E3, except for experiments using DHT, in which treatments were prepared in 0.08% (vol/vol) MeOH (vehicle) in E3. Zebrafish embryos were immersed in treatment within 3 hpf except where otherwise indicated. Embryos were treated with BPA [4,4′-(propane-2,2-diyl)diphenol] (239658; Sigma-Aldrich) prepared at concentrations of 0.0068, 0.05, 0.1, and 1 μM, BPS (4,4′-sulfonylbisphenol) (103039; Sigma-Aldrich) at a concentration of 0.0068 μM, and DHT (D-073 Cerilliant via Sigma-Aldrich) at a concentration of 1 μM individually or were coexposed with another pharmacological agent. Pharmacological drugs used were 1 μM ICI (I4409; Sigma-Aldrich), 1 μM FAD (fadrozole hydrochloride) (F3806; Sigma-Aldrich); 6.17 μM flutamide (F9397; Sigma-Aldrich), 0.1 μM GSK4716 (G6173; Sigma-Aldrich), and 50 nM amiodarone (amiodarone hydrochloride) (A8423; Sigma-Aldrich). Estradiol [(17β)-estra-1,3,5 (10)-triene-3,17-diol] (17β-estradiol; Sigma-Aldrich) treatment was prepared at a concentration of 0.0068 μM. In coexposure experiments, embryos were exposed to BPA, BPS, or DHT from 0–5 dpf and/or were coexposed to 1 μM ICI from 0–5 dpf, 1 μM FAD from 24–48 hpf (behavior assay) or from 0–5 dpf (neurogenesis assay), 6.17 μM flutamide from 8–48 hpf, 0.1 μM GSK4716 from 0–5 dpf, or 50 nM amiodarone from 0–5 dpf. At 5 dpf, embryos were assayed for neurogenesis or locomotor behavior (see below). In *cyp19a1b* qRT-PCR experiments, 0.0068 μM BPA, 1 μM DHT, 6.17 μM flutamide, and 1 μM ICI were administered from 8–48 hpf.

MO-AroB–injected embryonic zebrafish were exposed to 0.0068 μM or 1 μM BPA or 0.0068 μM BPS from 0–5 dpf. Chemical structures of BPA and BPS were designed using ChemBioDraw 13.0 (PerkinElmer) software.

MO Analyses. AroB morpholinos (MO-AroB) were designed and engineered by Gene-Tools (2). Sequences were verified for binding specificity using BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi). One MO was designed to bind the ATG translation site to prevent binding of the translational complex to processed *cyp19a1b* mRNA (5′-AGGCTTCCATCATCCCCAACTTCAT-3′). A second MO was designed to bind a splice junction preventing processing of pre-mRNA at exon 9 (5′-CGAGCCTGAGAGGACAACAAAGACA-3′). Both MOs were resuspended in 16 μL double-distilled H_2O and 2 μL phenol red to a final concentration of 20 nM MO (2). Embryos were injected with 2.6–4.6 nL of a 1:1 mixture of both MOs or vehicle control at the one-cell stage and were sorted for phenol red coloring in the developing embryo 3 h later. Embryos lacking a light red hue were discarded. Vehicle- and MO-injected embryos were immersed in their respective treatment until assayed for neurogenesis or locomotor behavior.

Locomotor Behavior Assays. Larval zebrafish were maintained in 96-well plates immersed in respective treatments from 0–5 dpf, similar to methods described by Saili, et al. (3).

For experiments with restricted temporal exposure to contaminants, embryonic zebrafish were exposed to vehicle only or were exposed to vehicle until 10 hpf, then were exposed to 0.1 μM BPA from 10–16 hpf, and then were returned to vehicle or were exposed to vehicle until 16 hpf, then were exposed to BPA from 16–24 hpf, and then were returned to vehicle, or were exposed to vehicle until 24 hpf, then were exposed to BPA from 24–36 hpf, and then were returned to vehicle. At 5 dpf, larvae were acclimated for 20 min in the light (100% intensity) in the Zebrabox (ViewPoint Life Sciences) and were monitored for locomotor activity for 5 min in the dark. Larval locomotor activities were tracked and analyzed using ZebraLab V3 software (ViewPoint Life Sciences). A difference of five or more pixels between two frames was set as the activity threshold. The duration of hyperactivity bursts (rapid and continuous turning during a singular swimming bout) (4) in seconds per minute above the threshold for 5 min was recorded and calculated for each larva.

Neurogenesis Assessments. Embryos were exposed to 0.0068 μM or 1 μM BPA, 0.0068 μM BPS, 1 μM DHT, or 0.0068 μM β-estradiol individually or were coexposed with a pharmacological agent. Embryos were pulsed with the S-phase marker 5-ethynyl-2’-deoxyuridine (EdU) (C10338; Molecular Probes) 0.01% (wt/wt) in their respective treatment at 9, 12, 24, 36, and 48 hpf, corresponding to the window of neurogenesis. EdU was washed off after 30 min total (15 min on ice and 15 min at room temperature), and embryos were replaced in their respective treatments until they were killed at 5 dpf by immersion overnight in 4% PFA in PBS (pH 7.4) at 4 °C. After sectioning and immunohistochemical processing, labeled hypothalamic brain sections on slides were imaged with the Zeiss Observer21 AX10 (Carl Zeiss). Images were adjusted for clarity, and cells were counted by three independent persons to ensure accuracy using Adobe Photoshop 6 Extended (Adobe Systems). To ensure that BPA-treated cells did not differ in size or shape relative to control, cell morphology (e.g., area) was measured using Adobe Photoshop 6 Extended and was compared between treatment groups. In addition, the total number of cells within the measurement field of view was compared within each treatment group to safeguard against variation in brain region size caused by BPA exposure.

Sectioning and Immunohistochemistry. After overnight fixation at 4 °C with 4% PFA, larvae were washed three times for 10 min each washing with PBS containing 0.1% Triton X-100 (234729; Sigma-Aldrich) (PBT) and then were cryoprotected in 30% sucrose in diethylpyrocarbonate-PBS overnight. Larvae then were embedded in Clear Frozen Section Compound (CA95057-838; VWR Scientific), snap frozen, and kept at −80 °C for future use. Embedded larvae were sectioned at 10 μm with a Leica CM3050 S Cryostat (Leica Microsystems). Slides were dried, and sections throughout the hypothalamic area were identified by referencing Mueller and Wullimann (5). Protein-binding sites were unmasked via antigen retrieval with a citrate buffer [10 mM sodium citrate (pH 6.0) + 0.5% Tween-20] by boiling for 20 min. Following one 10-min wash with PBT and a 30-min permeabilization step with PBS 1% Triton X-100, slides were incubated with blocking buffer (block) consisting of 5% normal goat serum (S1000; Vector Laboratories) in PBT for 1 h. Slides then were left overnight in block with the general neuronal marker (primary antibody), mouse anti-human neuronal protein HuC/HuD (α-HuC/D) (A21271; Molecular Probes; 1 block: 400 HuC/HuD). After four 10-min washes with PBT, slides were treated in block with the secondary antibody, Alexa Fluor 488 goat α-mouse IgG (A11001; Molecular Probes; 1:400), for 2 h. Following four 10-min washes with PBT, slides were treated DAPI (D1308; Molecular Probes; 1:1,000) for

5 min and then were washed three times with PBT. Next, a Click-iT EdU Alexa Fluor 555 Imaging Kit (C10338; Molecular Probes) was used to label DNA-integrated EdU for 2 h, followed by three 10-min washes with PBT. Slides then were mounted with Vectashield Mounting Medium for fluorescence (H-1000; Vector Laboratories) in preparation for imaging. Binding specificity of α-HuC/D was tested by the omission of primary antibody α-HuC/D on a slide containing hypothalamic sections, and the specificity of EdU was confirmed by omitting the EdU pulse on a subset of fish.

Transcript Measurement Assays. qRT-PCR was performed using a BioRad iCycler on pools of 10 treated larvae at 48 hpf following the protocol in Kurrasch et al (6). The qPCR reaction was carried out as follows: initial denaturation at 95 °C for 4 min, 40 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1.5 min. BPA, DHT, and pharmacological coexposures were applied from 8–48 hpf. For MO validation studies, RT-PCR sample preparation was conducted largely as described above and previously (6); AroB-MO–injected and vehicle-injected 48-hpf embryos were ground, and mRNA was extracted. PCR reactions were carried out using the following cycles on Mastercycler Pro (Eppendorf): initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 68 °C for 1.5 min. PCR products were analyzed on a 2% ethidium bromide gel using agarose gel electrophoresis and were compared. Primer sequences are given in Table S1.

Statistical Analyses. Assumptions of normality and equality of variance were verified using the D'Agostino and Pearson omnibus normality test and the Brown–Forsythe test, respectively. Results from qRT-PCR experiments were log transformed before statistical analyses. ANOVA followed by Tukey's Honestly Significant Difference test and Student's *t* test were performed where indicated. All analyses were performed using Prism 6 (GraphPad Software).

SI Supporting Notes

Rationale for Chemical Treatment Concentrations. To position our paper in the context of previous work, the concentrations for pharmacological agents used were selected in accordance with those cited in peer-reviewed literature (see main text for specific references). For example, the flutamide dose used is in the midrange of chemical concentrations reported in zebrafish (7) and ricefield eel (8) publications, and the efficacy of AR antagonization has been verified with minimal to no off-target effects. Given that BPA conventionally is thought to bind ERs, we thought it necessary to test the efficacy of the dose of the ER antagonist ICI 182, 780 used here (Fig. S2).

Rationale for Exposure Schedules. Results of locomotor activity assays are shown in Figs. 1 and 6 *C* and *D*. To capture the behavioral effects of BPA or BPS later in life, locomotor activity assays were performed at 5 dpf, a time point when embryogenesis is complete and larval zebrafish are able to respond to stimuli, launch a stress response, and actively seek prey. In Fig. 1*A*, we show that restricted exposure to BPA during the period of neurogenesis is sufficient to induce increased locomotor activity (Fig. 1*A*). Moving forward we strategically opted to expose zebrafish to most compounds from 0–5 dpf, given that handling zebrafish invokes a stress response to which the hypothalamus responds. Thus, we sought to limit stress effects as a confounding variable. We confirmed this strategy by showing that BPA-mediated effects were blocked only from 24–48 hpf by FAD (an aromatase inhibitor) treatment, despite continuous exposure to BPA from 0–5 dpf (Fig. 1*F*).

For neurogenesis assays (Figs. 2, 5 *A–C*, and 6*B*), zebrafish were exposed to BPA or BPS, with/without pharmacological agents from 0–5 dpf and were pulsed with EdU at 24 and 36 hpf. Because these birthdating studies measure the neurons born at or shortly (within the hour) after the time point of the EdU pulse, any BPA (or BPS/pharmacological agent) effect outside that window would not be captured in our assay. Thus, we kept treatment paradigms consistent with the behavioral assays and dosed developing zebrafish from 0–5 dpf.

For the transcript assay (Fig. 5*D*, *cyp19a1b*), qRT-PCR experiments were conducted on zebrafish exposed to BPA and/or pharmacological agents from 8–48 hpf (Fig. 5*D*). In this experiment, we show that flutamide exposure during the restricted window of 8–48 hpf was sufficient to attenuate up-regulation of *cyp19a1b* expression, providing further evidence that the neurogenic phase represents a window of vulnerability.

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2. Summerton J (1999) Morpholino antisense oligomers: the case for an RNase H-independent structural type. *Biochim Biophys Acta* 1489(1):141–158.
3. Saili KS, et al. (2012) Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish. *Toxicology* 291(1-3):83–92.
4. Kalueff AV, et al.; Zebrafish Neuroscience Research Consortium (2013) Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish* 10(1):70–86.
5. Mueller T, Wullimann MF (2005) Atlas of Early Zebrafish Brain Development: A Tool for Molecular Neurogenetics (Elsevier, Amsterdam) 1st Ed. p 183.
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7. Schiller V, et al. (2013) Transcriptome alterations in zebrafish embryos after exposure to environmental estrogens and anti-androgens can reveal endocrine disruption. *Reprod Toxicol* 42:210–223.
8. Zhang Y, et al. (2012) Androgen rather than estrogen up-regulates brain-type cytochrome P450 aromatase (cyp19a1b) gene via tissue-specific promoters in the hermaphrodite teleost ricefield eel Monopterus albus. *Mol Cell Endocrinol* 350(1): 125–135.

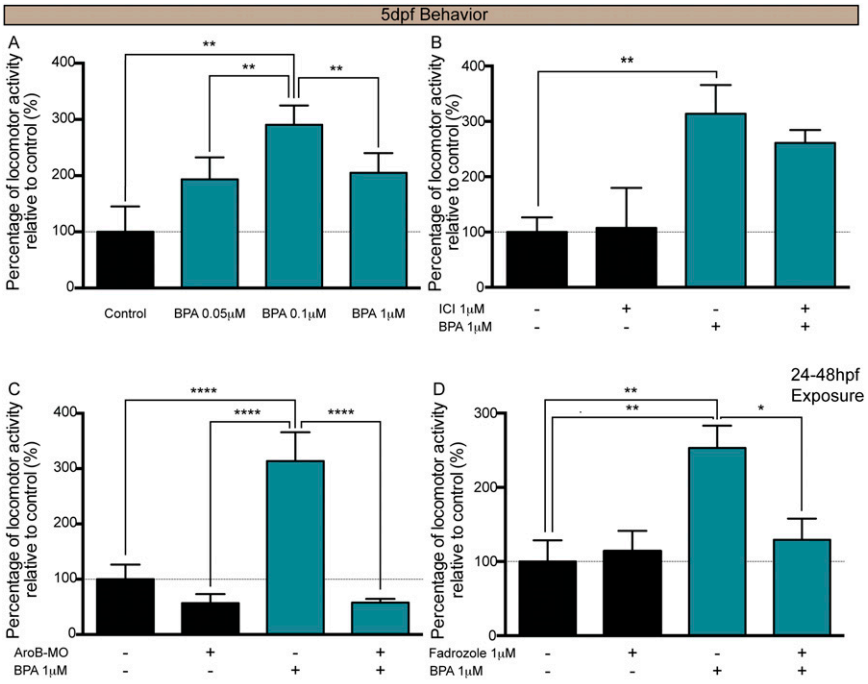

Fig. S1. Greater doses of BPA induce hyperactive behavior via an AroB-mediated mechanism. (*A*) BPA-induced locomotor activity in 5-dpf zebrafish compared with vehicle control is shown for exposure from 0–5 dpf to doses of 0.05, 0.1, or 1 μM BPA. (*B*) Locomotor activity in 5-dpf zebrafish coexposed from 0–5 dpf to a higher (1 μM) dose of BPA + the ER inhibitor ICI (1 μM). (*C*) Locomotor activity in 5-dpf zebrafish coexposed from 0–5 dpf to AroB morphants ± 1 μM BPA. (*D*) Locomotor activity in 5-dpf zebrafish exposed to 1 μM BPA ± 1 μM FAD from 24–48 hpf. (*B* and *C*) BPA exposure of AroB morphants and BPA+ICI treatment run in same experiment. Results have been separated for simplicity (i.e., control and BPA values are the same). Data in *A–D* are shown as mean ± SEM; $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ (ANOVA, Tukey's HSD); $n = 5–10$ fish.

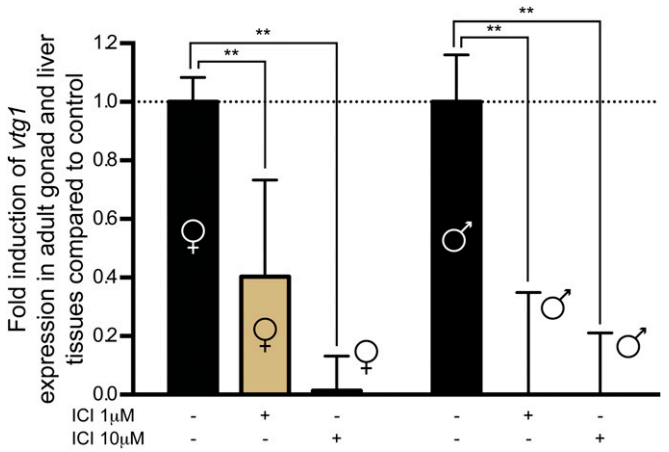

Fig. S2. Validation of the ICI dose. Demonstration that the ICI dose used is sufficient to interfere with transcription of estrogen-target genes. *vtg1* expression measured by qRT-PCR in pooled liver and gonad samples from adults after exposure to either 1 μM or 10 μM ICI for 96 h. Data shown as mean ± SEM; $**P < 0.01$. (ANOVA, Tukey's HSD); $n = 3–5$ fish.

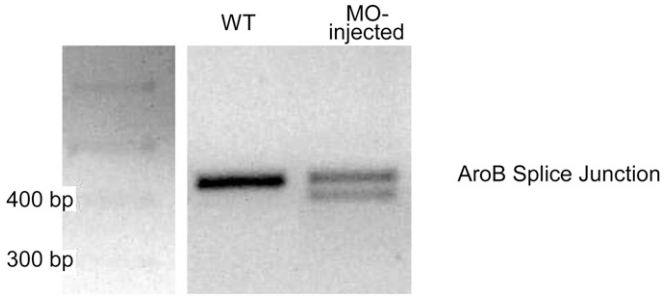

Fig. S3. Validation of AroB-MO. Demonstration of AroB-MO efficacy. RT-PCR spanning the exon 9 splice junction in wild-type and AroB morphants at 48 hpf. Alternative splicing of the AroB transcript in MO-injected embryos is shown. Of note, all experiments throughout the paper were conducted with a 1:1 mix of ATG- and splice junction-blocking AroB MOs, so knockdown presumably is significantly higher than represented by this RT-PCR, as suggested by the tight error bars in neurogenesis and locomotor activity assays. ATG-blocking MO could not be verified by Western blot because of poor zebrafish AroB antibodies.

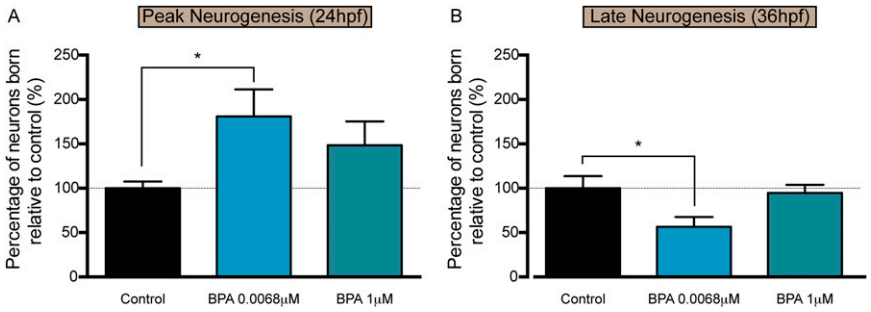

Fig. S4. Exposure to low-dose but not to moderate-dose BPA induces precocious neurogenesis. (*A*) Quantification of neuronal birth at 24 hpf in 5-dpf zebrafish exposed from 0–5 dpf to a higher (1 μM) dose of BPA. (*B*) Quantification of neuronal birth at 36 hpf in 5-dpf zebrafish exposed from 0–5 dpf to a higher (1 μM) dose of BPA. Data are shown as mean ± SEM; $*P < 0.05$ (ANOVA, Tukey's HSD); $n = 6$ or 7. from 0–5 dpf.

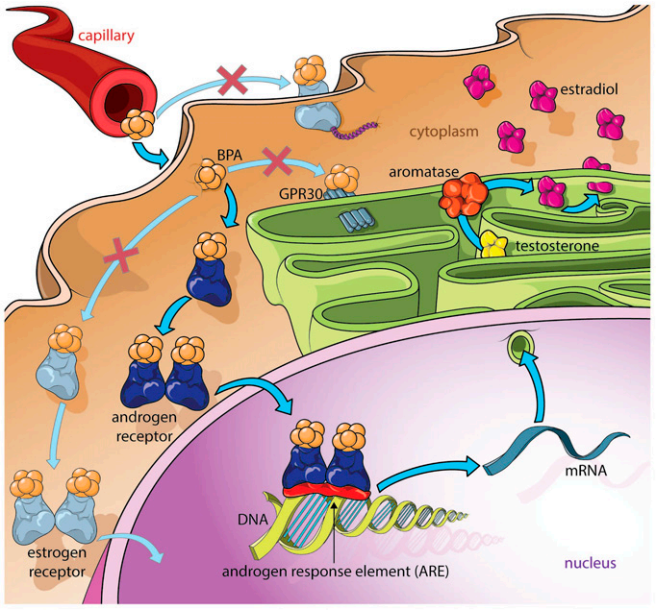

Fig. S5. Model of BPA-mediated increases in aromatase expression through AR agonism. BPA readily diffuses across the progenitor plasma membrane and binds ARs, perhaps causing receptor dimerization. After translocation into the nucleus, the BPA–AR complex interacts with the ARE present in the AroB (*cyp19a1b*) promoter, increasing expression of AroB (*cyp19a1b*) transcripts. Once in the smooth endoplasmic reticulum, AroB catalyzes the conversion of testosterone into estradiol, which then might diffuse into the cytoplasm and influence local intracellular signaling pathways. We hypothesize that local estrogen synthesis plays a key role in inducing precocious neurogenesis in hypothalamic progenitors in embryonic zebrafish and that this precocious neurogenesis then leads to hyperactive behaviors in zebrafish larva. Treatment with a broad ER ligand (ICI) had no effect on neurogenesis or locomotor behavior, and treatment with an AR antagonist (flutamide) attenuated BPA-mediated precocious neurogenesis, suggesting that BPA acts through the AR to modulate hypothalamic neurogenesis. Coexposure to BPA + flutamide and ICI significantly attenuated increased aromatase (*cyp19a1b*) expression, suggesting that BPA may bind both ARs (ARα and ARβ) and ERs [genomic (ERα, ERβ) or nongenomic (mER, GPR30)] to increase aromatase expression in hypothalamic progenitors. However, BPA neurogenic effects likely are restricted to AR signaling.

Table S1. qRT-PCR and RT-PCR primer sequences

Gene	Forward (5′–3′)	Reverse (3′–5′)
qRT-PCR		
*cyp19a1b**	AAAGAGTTACTAATAAAGATCCACCGGTAT	TCCACAAGCTTTCCCATTTCA
vtg1	CAACGGAAACGCTCATTGC	CAACGGAAACGCTCATTGC
β-actin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
RT-PCR		
Cyp19a1b spanning exon 9 (MO validation)	CAGTGTGTGCTGGAGATGGT	ACAACCGAATGGCTGGAAGT

*Sawyer SJ, Gerstner KA, Callard GV (2006) Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: Gene specific tissue distribution, sex differences, developmental programming, and estrogen regulation. *Gen Comp Endocrinol* 147(2):108–117.