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Actions of Bisphenol A and Bisphenol S on the Reproductive Neuroendocrine System During Early Development in Zebrafish

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Bisphenol A (BPA) is a well-known environmental, endocrine-disrupting chemical, and bisphenol S (BPS) has been considered a safer alternative for BPA-free products. The present study aims to evaluate the impact of BPA and BPS on the reproductive neuroendocrine system during zebrafish embryonic and larval development and to explore potential mechanisms of action associated with estrogen receptor (ER), thyroid hormone receptor (THR), and enzyme aromatase (AROM) pathways. Environmentally relevant, low levels of BPA exposure during development led to advanced hatching time, increased numbers of GnRH3 neurons in both terminal nerve and hypothalamus, increased expression of reproduction-related genes (*kiss1*, *kiss1r*, *gnrh3*, *lhβ*, *fshβ*, and *erα*), and a marker for synaptic transmission (*sv2*). Low levels of BPS exposure led to similar effects: increased numbers of hypothalamic GnRH3 neurons and increased expression of *kiss1*, *gnrh3*, and *erα*. Antagonists of ER, THRs, and AROM blocked many of the effects of BPA and BPS on reproduction-related gene expression, providing evidence that those three pathways mediate the actions of BPA and BPS on the reproductive neuroendocrine system. This study demonstrates that alternatives to BPA used in the manufacture of BPA-free products are not necessarily safer. Furthermore, this is the first study to describe the impact of low-level BPA and BPS exposure on the Kiss/Kiss receptor system during development. It is also the first report of multiple cellular pathways (ERα, THRs, and AROM) mediating the effects of BPA and BPS during embryonic development in any species. **(*Endocrinology* 157: 636–647, 2016)**

Bisphenol A (BPA), a well-known endocrine disrupting chemical, is a constituent of plastic polycarbonate and epoxy resins, which are widely used to produce plastic food and beverage containers, paints, adhesives, drinking water pipe linings, dental sealants, and household paper products. To meet these manufacturing needs, 2.7 billion kilograms of BPA were produced worldwide in 2008 and 5.5 billion kilograms were projected to be produced in the United States and Europe by 2015 (1, 2). The total release of BPA to the environment was about 2.5 million kilograms in 2007, with direct release of 13 772 kilograms to water (3). According to studies from the United States, China, Japan, Germany, The Netherlands, and Spain, BPA levels in river water were 8 μg/L or less but reached as high as 21 μg/L (4). Also, studies have determined that BPA can be measured in human serum, urine, umbilical cord blood, amniotic fluid, and placental tissue (5–7). Serum concentrations of BPA were found to covary with sex steroid hormone levels in humans, suggesting that BPA could have an effect on human reproductive function, specifically by altering circulating steroid hormones (8, 9). As a consequence of its ubiquitous existence in the environment, wide detection in human biological samples, and extensive application in our daily lives, BPA has raised

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Abbreviations: AMIO, amiodarone hydrochloride; AROM, aromatase; BPA, bisphenol A; BPS, bisphenol S; DMSO, dimethyl sulfoxide; E2, estradiol; EE2, 17α-ethynylestradiol; ER, estrogen receptor; FAD, fadrozole hydrochloride; hpf, hours post fertilization; HYPO, hypothalamus; ICI, ICI 182780; Kiss, kisspeptin; Kissr, kisspeptin receptor; LC-MS/MS, liquid chromatography-tandem mass spectrometry; qPCR, quantitative PCR; sv2, synaptic vesicle protein-2; THR, thyroid hormone receptor; TN, terminal nerve.

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considerable public health concerns (10). As a result, some regulatory agencies, such as the European Commission (11), the US Food and Drug Administration (12), and Health Canada (13), have banned the use of BPA during recent years in baby bottles. Given these restrictions and societal pressure, manufacturers seeking BPA alternatives have turned to alternative chemicals to produce BPA-free products (14).

Bisphenol S (BPS) is one of the BPA-alternative chemicals, and its production is increasing annually (15). Based on previous studies, BPS was considered a safer alternative to BPA because of its significantly lower estrogenic activity and excellent stability against high temperature and resistance to sunlight (16–18). BPS has been detected in sediment samples (19), canned foodstuffs (20), thermal paper receipts (14), and human urine (21). Recent work in zebrafish reported potential effects of BPS on endocrine and reproductive functions. Ji et al (22) showed that exposure to low levels of BPS could affect the feedback regulatory circuits of the hypothalamo-pituitary-gonadal axis and impair offspring development. Additional work suggests that developmental exposure to environmentally relevant concentrations of BPS advances hypothalamic neurogenesis in embryonic zebrafish (23) and has adverse effects on different parts of the endocrine system in adult zebrafish (24).

GnRH, a decapeptide hormone synthesized in the hypothalamus, is critical for normal vertebrate reproductive function by stimulating the release of FSH and LH from the anterior pituitary, which in turn regulate gonadal functions (25). Moreover, the neuropeptide kisspeptin (Kiss) and its receptor GPR54 (now known as Kiss receptor [Kissr]) have been shown to play important roles in regulating GnRH neuron biology in mammals (26, 27). Thus, BPA and other endocrine disrupting chemicals could potentially alter the hypothalamo-pituitary-gonadal axis, in part, by affecting the GnRH and/or Kiss systems. Previous studies have shown that BPA elicits weak estrogenic activity, alters release of GnRH and Kiss and interferes with neuroendocrine function. Specifically, acute BPA exposure at the level of 10 nM was found to suppress both GnRH and Kiss hypothalamic release in female rhesus monkeys (28). Furthermore, treatment of male rats with an environmentally relevant dose of BPA from birth until 50 and 90 days increased the numbers of anteroventral periventricular Kiss neurons and GnRH-immunoreactive cells (29). Similar to mammals, zebrafish express multiple forms of GnRH, including GnRH2 and GnRH3 (but not GnRH1). In zebrafish, GnRH3 is considered to be the hypophysiotropic form of GnRH based on the localization of GnRH3 neurons in the hypothalamus/preoptic area and high density of GnRH3 nerve terminals in the anterior pituitary (30). Zebrafish has become an important animal model for understanding the molecular and cellular regulation of GnRH neuron development and physiology (30, 31). However, relatively few studies have explored the potential neuroendocrine impact of environmentally relevant levels of BPA and BPA analogs in aquatic animals, including zebrafish. The present study takes advantage of using a unique transgenic zebrafish model system in which GnRH3 neurons are genetically tagged with a bright variant of green fluorescent protein. This allows us to visualize GnRH3 morphological changes in the intact brain during early embryonic development (32).

BPA has a relatively weak estrogenic potency compared with natural estradiol (E2) based on its binding affinity and activating capacity on estrogen receptors (ERs) (33). ERs are expressed in developing hypothalamic cells and participate in the regulation of the neuroendocrine response (34). Thus, xenoestrogens like BPA and BPS may target neuroendocrine systems and modulate their neurosecretory competence by acting as weak ER agonists (35). For the purposes of the present study using zebrafish as a model system, it is notable that BPA has been shown to activate ERs in several teleost species (36–38).

BPA is also thought to exert its effects via thyroid hormone receptor (THR) and the aromatase (AROM) pathways because it induces strong brain-specific overexpression of thyroid receptors and aromatase (39, 40). The objective of the present study is to determine the impact of BPA and BPS on the reproductive neuroendocrine system during development and to reveal possible cellular pathways mediating those effects. We investigated the effects of a range of BPA concentrations on embryo survival, hatching rate, and GnRH3 neuron numbers in the terminal nerve (TN) and hypothalamus (HYPO) as well as the expression of reproductive-neuroendocrine related genes including *kisspeptins 1* and *2* (*kiss1* and *kiss2*), *kiss1r, kiss2r, gnrh3, synaptic vesicle protein-2* (*sv2*), *lhβ*, *fshβ*, *erα*, and *erβ* during embryonic and early larval development. In addition, we investigated the potential roles of ERs, THRs, and AROM in mediating the effects of BPA on the reproductive neuroendocrine system. We also studied whether BPS, a replacement for BPA that is thought to be safer, caused alterations in normal development of the reproductive neuroendocrine system in a similar manner as BPA. The present study adds critical evidence to a small body of work indicating that exposure to BPS is not necessarily benign.

Materials and Methods

Chemicals

BPA (Chemical Abstracts Service number 80–05-7, 99+%) and BPS (Chemical Abstracts Service number 80–09-1, 98+%)

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were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions of 10 g/L each and stored at 4°C. Fresh stock solutions were made every week. The final working solutions of BPA and BPS contained no more than 0.005% DMSO. Unless otherwise noted, all chemicals used were of analytical grade and purchased from Sigma-Aldrich.

Animals

We used a transgenic zebrafish in which the GnRH3 promoter drives the expression of a bright variant of green fluorescent protein (Emerald green fluorescent protein or EMD) to easily identify GnRH3 neurons starting from early embryonic development (32). This was important for the confocal experiments described below; for consistency, we used these transgenic fish in all experiments. Brass GnRH3-EMD transgenic zebrafish were maintained in flow-through aquarium systems on a 14-hour light, 10-hour dark photoperiod at 28°C ± 0.5°C. The fish were fed with flake food and live brine shrimp (*Artemia nauplii*) twice daily. Fertilized eggs were collected within 1 hour after adult spawning. The embryos were kept in a 28°C incubator and held in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.3 mM $MgSO_4$). Test solutions (composition described below) were made in E3 medium. Both control and test solutions were replaced completely every 12 hours. Zebrafish have no identified sex chromosomes and cannot be sexed until gonad differentiation occurs (between 35–45 d after fertilization). All of the experiments were performed in embryos and larvae that have not yet sexually differentiated. All procedures were carried out in accordance with and approved by the Animal Care and Use Committee of the University of California, Los Angeles.

Experimental design

Zebrafish embryos (2 h post fertilization [hpf]) from three to five male/female mating pairs were randomly distributed in petri dishes (100 embryos per dish) just prior to exposure to test solutions (50 mL). The embryos were exposed to BPA at concentrations of 0.1, 1, 10, 100, and 1000 μg/L in E3 medium starting at 2 hpf until either 25 or 120 hpf. Hatching rate, an important index for evaluation of embryonic development, defined as the percentage of hatched embryos to all survived ones, was recorded at 48 hpf and 55 hpf. Survival rate, defined as the percentage of embryos that survived under all treatment conditions, was recorded every 24 hours for 120 hours.

The forebrain populations of GnRH3 neurons were analyzed by confocal microscopy during early embryogenesis at 25 hpf (n = 14 embryos per treatment group), and neuroendocrine-related gene expression was measured at both 25 hpf and 120 hpf (20 embryos per treatment group; n = 6 replicate experiments). Later experiments compared the effects of BPA and BPS using the outcome from the previous study. Embryos were exposed to 100 μg/L BPA and 100 μg/L BPS for 25 hpf. This is the dose of BPA that had consistent effects on GnRH3 neuron number and reproductive-related gene expression (Figure 1, B, C, and D). As with the previous study, forebrain populations of GnRH3 neurons were analyzed by confocal microscopy (n = 14 embryos per treatment group) and neuroendocrine-related gene expression was measured (20 embryos per treatment group; n = 6 replicate experiments).

The final set of experiments explored potential signal transduction pathways, including ERs, THRs, and AROM that converts androgen to estrogen, which might mediate the actions of BPA and BPS on neuroendocrine-related gene expression. Embryos (50 embryos per treatment group; n = 6 replicate experiments) were exposed for 120 hours to 100 μg/L BPA or 100 μg/L BPS and in the presence or absence of the ER antagonist ICI 182780 (ICI; 1 μM), the THR antagonist amiodarone hydrochloride (AMIO; 1 μM), or the AROM inhibitor fadrozole hydrochloride (FAD; 1 μM). The concentrations of these antagonists/inhibitors are according to previous work in zebrafish by Kinch et al (23). ICI can have partial agonist and antagonist actions depending on the system studied. In zebrafish, it has been shown to act as an antagonist of all three ERs: esr1, esr2a, and esr2b (41). If any of these inhibitors block the actions of BPA or BPS, it would suggest which cellular pathways mediate the effects of these endocrine-disrupting chemicals on expression of the reproductive-neuroendocrine related genes investigated.

Measurement of BPA in test solutions

The test solutions were collected after a 12-hour exposure to zebrafish embryos and filtered through 0.45-μm membrane filters (Anpel). The exposure concentrations of BPA were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Agilent 1260 Infinity liquid chromatograph equipped with a Poroshell 120 EC-C18 column (3 × 100 mm, 2.7 μm) and an Agilent 6460 triple-quadrupole mass spectrometer. Sample injection volume was 5 μL and the flow rate was 0.4 mL/min. The mobile phases started with 5% methanol and 95% water. The gradient was increased to 90% methanol at 0.75 minutes and then held for 4 minutes. The tandem mass spectrometry parameters are as follows: capillary voltage of 2500 V, nebulizing pressure of 35.0 psi, sheath gas temperature at 350°C, sheath gas flow at 6 L/min. The mass spectrometer was operated with the following ion transition: BPA (227.2→212.2). The detection limit of the assay was 2.30 ng/L. Because BPS has been shown to be more stable than BPA (16), we did not measure its potential degradation.

Confocal microscopy

Transgenic embryos (25 hpf) expressing the GnRH3-EMD gene were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C for morphological analysis of GnRH3-EMD neurons. Fixed embryos were mounted in 0.8% agarose in the ventral-side up position to best observe both TN and HYPO populations of GnRH3 neurons. Images were taken and analyzed under an upright Olympus confocal microscope using a water immersion 40× objective lens and Fluoview software (Olympus America Inc). An Argon laser (488 nm) with an emission barrier filter of 510 nm was used to detect EMD fluorescence. Optical sections were made at an interval of 1 μm along the z-axis. Following image analysis, the number of GnRH3-EMD neurons in TN and HYPO were analyzed blind to the treatments.

RNA isolation, reverse transcriptase, and quantitative PCR (qPCR)

Total RNA was extracted from the homogenates of 20 zebrafish larvae from each sample pool using the Quick-RNA miniprep kit (Zymo Research), following the manufacturer's protocol. Reverse transcriptase reaction was performed from 1000 ng of total RNA in a final volume of 20 μL using the

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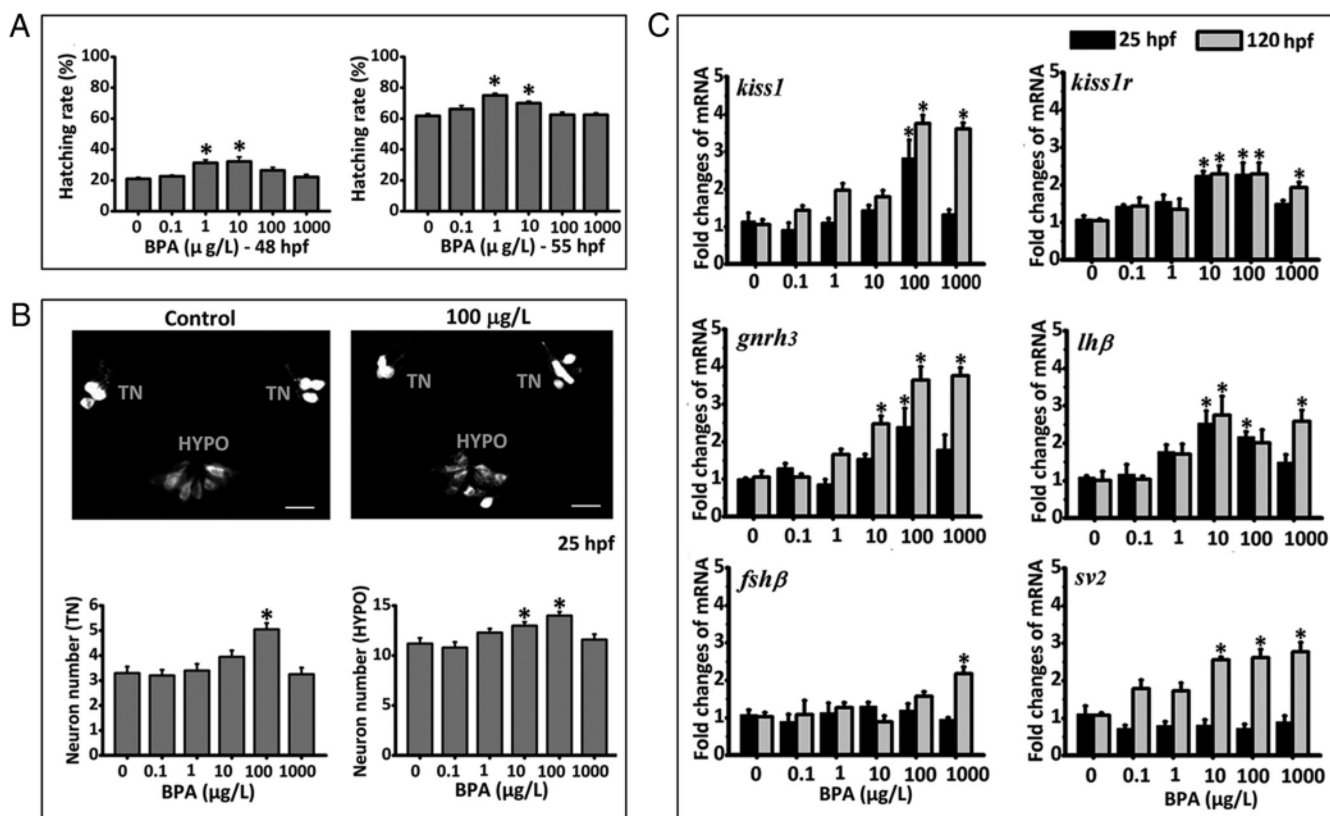

Figure 1. Effects of BPA on hatching rate measured at 48 and 55 hpf (A), GnRH3 neuron numbers in zebrafish embryos after 25 hpf (B), and expression of reproductive neuroendocrine-related genes in zebrafish embryos (25 hpf) and larvae (120 hpf) for *kiss1*, *kiss1r*, *gnrh3*, *lhβ*, *fshβ*, and *sv2* (C). B, Upper panel, Z-stack of confocal images of ventral view of terminal nerve GnRH3-EMD neurons (TN) and hypothalamic GnRH3-EMD neurons (HYPO) of a representative control embryo and embryo treated with 100 μg/L BPA. Horizontal bars indicate 20 μm. B, Lower panel, Summary data of the numbers of TN-GnRH3 and HYPO-GnRH3 neurons after exposure to increasing concentrations of BPA. Values are shown as mean ± SE (n = 6 each for hatching rate; n = 14 each for GnRH3 neuron numbers; n = 6 replicate experiments each for mRNA levels). Asterisks indicate significant difference compared with control at $P < .05$ (ANOVA, Dunnett's test).

high-capacity RNA-to-cDNA kit (Life Technologies). Quantitative PCR was performed using the SYBR Green PCR master mix kit (Life Technologies) and Mx3000P qPCR system (Agilent Technologies). The reaction mixture consisted of 1 μL cDNA, 10 μL 2× qPCR mixture, and the appropriate forward and reverse primers to a final volume of 20 μL. Quantitative PCR conditions were set as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescent signals were measured at the annealing/extension step. Melting curve analyses were performed to validate the specificity of PCR amplicons. Ribosomal protein L13A (*rpl13a*) was used as an internal reference gene for each tested sample according to previous study (42). A cycle threshold-based relative quantification with efficiency correction normalizing to *rpl13a* was calculated using the $2^{-\Delta\Delta Ct}$ method. For each tested gene, RT-qPCRs were performed in duplicate on three sets of samples. The primers specific for target genes and the *rpl13a* are listed in Table 1. We followed the convention of gene and protein nomenclature guidelines for fish as described in Genetics and Molecular Biology (43).

Statistical analysis

Data are shown as mean ± SEM. In all experiments, intergroup differences were assessed by using a one-way ANOVA, followed by either Dunnett's or Duncan's tests, depending on the experimental design. For all the tested parameters, there was no significant difference between a blank control group (no DMSO) and the vehicle control group (with DMSO), and thus, the vehicle control was set as the control group for the statistical analysis that followed. The level for statistical significance was set at $P < .05$, indicated by an asterisk in the figures. The correlations between measured parameters were analyzed according to Pearson's test and the level for statistical significance was set at $P < .05$, indicated by an asterisk in Supplemental Tables 1 and 2. Statistical analyses used SPSS 18.0 software (SPSS, Inc, Chicago, IL, USA).

Results

Exposure concentrations of BPA

There is concern that BPA might degrade during the course of treatment. To confirm the concentrations of BPA during treatment, BPA levels in exposure solutions were measured using LC-MS/MS. Measured exposure concentrations deviated less than 15% from the nominal concentrations of BPA (Table 2). BPA levels were undetectable in the control solution. These results indicate that

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Table 1. The DNA Sequences of Primers Used for qPCR

Name	Primers		GenBank
	Forward (5′–3′)	Reverse (5′–3′)	
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	NM_212784
kiss 1	ACAAGCTCCATACCTGCAAGTG	AATACTGAAAATGCCCAGAGGG	AB245404
kiss 2	GCCTATGCCAGACCCCAAA	TTTACTGCGTGCTAGTCGATGTTT	AB439561
gnrh3	TTGCCAGCACTGGTCATACG	TCCATTTCACCAACGCTTCTT	AY657019
sv2c	TTCAAGCTCTGGATGGCTGG	GATGCTGTGGTTATCTGACCG	NM_001128339
kiss1r	TCAACAGGTGACGGTACAGG	ATGGTGCAGGGATTTGAGAG	AY860977
kiss2r	CCGTTCAAGGCTGTAACCAG	CAGCACCATCACCACTACCA	NP[a]
lhβ	GCAGAGACACTTACAACAGCC	AAAACCAAGCTCTGAGCAGCC	AY424304
fshβ	TGAGCGCAGAATCAGAATG	AGGCTGTGGTGTCGATTGT	AY424303
erα	AAACACAGTCGGCCCTACAC	GCCAAGAGCTCTCCAACAAC	AF349412
erβ	TGATTAGCTGGGCGAAGA	TATCCAGCCAGCAGCATT	AJ141566

Abbreviation: NP, not provided.

[a] Sequences of *kiss2r* was not provided in GenBank; it was according to a previous study by Servili et al (27).

BPA did not degrade to an appreciable level under the conditions of these experiments.

Hatching and survival rates

The normal time of hatching of zebrafish embryos occurs between 2 and 3 days after fertilization. We measured the hatching rate of each group at 48 and 55 hpf. Comparisons were made between controls and the different doses of BPA at each of the two time points. We observed a significantly higher hatching rates at both time points in embryos upon exposure to environmentally relevant concentrations of 1 and 10 μg/L BPA, compared with the controls (ANOVA, $P < .001$; Dunnett's test, $P < .05$) (Figure 1A). Higher concentrations of BPA had no effect on hatching rates compared with controls. At 72 hpf, all the embryos in the control group had hatched. After exposure for 120 hours, there was no significant difference in survival rate among embryos treated with 0, 0.1, 1, 10, 100, and 1000 μg/L BPA (data not shown). The results indicate that exposure to low levels of BPA significantly advanced the time of hatching without impacting embryo survival.

Effects of BPA on GnRH3 neuron numbers in TN and HYPO

We investigated the effects of BPA on the development of GnRH3 neurons by analyzing the number of GnRH3 neurons in the forebrain of embryos. As shown in Figure 1, B and C, the numbers of GnRH3 neurons in TN and HYPO were increased after BPA exposure. Specifically, 100 μg/L BPA increased TN-GnRH3 neuron numbers (ANOVA, $P = .003$; Dunnett's test, $P < .05$), and both 10 and 100 μg/L increased HYPO-GnRH3 neuron numbers as compared with controls (ANOVA, $P = .004$; Dunnett's test, $P < .05$) at 25 hpf (Figure 1C).

Effects of BPA on expression of reproductive neuroendocrine-related genes

The effects of BPA treatment (100 μg/L) on the expression levels of several reproductive neuroendocrine-related genes were analyzed at 25 and 120 hpf to determine the potential impact of BPA on the early developing hypothalamo-anterior pituitary axis. Figure 1D shows relative levels of *kiss1*, *kiss1r*, *gnrh3*, *lhβ*, *fshβ*, and *sv2* mRNAs. With the exception of *fshβ* and *sv2*, all of the other mRNAs showed a significant increase in response to 10 and/or 100 μg/L BPA treatment at 25 hpf. We included *sv2* analysis because of our earlier work showing that this marker for synaptic transmission is expressed on GnRH3 neurons during embryonic development (44). Interestingly, the highest concentration of BPA (1000 μg/L) had no effect on expression levels any of the mRNAs at 25 hpf (Figure 1D). A similar pattern of gene expression responses was seen at

Table 2. BPA Concentrations in the Test Solutions as Measured by LC-MS/MS

Nominal Concentrations, μg/L	Measured Concentrations After Exposure for 12 h, μg/L (n = 6)	Percentage Change Compared With Nominal Concentration, %
0.1	0.110 ± 0.00661	110
1	0.890 ± 0.267	89.0
10	8.57 ± 1.37	85.7
100	93.0 ± 12.0	93.0
1000	947 ± 147	94.7

The detection limit of the assay was 2.30 ng/L.

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the older developmental time of 120 hpf, additionally with *fshβ* (ANOVA, $P = .005$; Dunnett's test, $P < .05$) and *sv2* (ANOVA, $P < .001$; Dunnett's test, $P < .05$) mRNAs now showing significant increases in expression in response to BPA. In contrast to 25 hpf, treatment with the highest dose of BPA (1000 μg/L) at 120 hpf increased the expression of *kiss1*, *kiss1r*, *gnrh3*, *lhβ*, *fshβ*, and *sv2*. Unlike *kiss1 and kiss1r*, *kiss2* and *kiss2r* expression showed no significant change in response to any concentration of BPA at either time points (data not shown). The correlations between GnRH3 neuron numbers in TN and HYPO as well as expression levels of reproductive neuroendocrine-related genes at 25 and 120 hpf were analyzed according to Pearson's test (SPSS 18.0) (Supplemental Tables 1 and 2). Our analyses indicated a statistically significant relationship of most tested parameters at both 25 and 120 hpf. Notably, we observed that *kiss1* and *gnrh3* mRNA levels at 120 hpf significantly correlated with expression levels of all other neuroendocrine-related genes ($P < .05$). Together all these results suggest Kiss1 is the primary regulator of the hypothalamo-pituitary axis in zebrafish under normal conditions.

BPS has similar effects as BPA on the early development of GnRH3 neurons and reproductive neuroendocrine-related gene expression

To evaluate the impact of BPS exposure on the zebrafish embryo, the concentration of 100 μg/L BPS was chosen to compare the effects with the same concentration of BPA as used in the above experiments. The survival rate of zebrafish embryos was not significantly altered in response to treatments (data not shown). The effects of BPA and BPS on GnRH3 neuron numbers in TN and HYPO and reproductive neuroendocrine-related genes were examined at 25 hpf. As shown in Figure 2A, the number of HYPO-GnRH3 neurons was significantly increased in response to BPS, in a similar manner as with exposure to BPA. However, BPS had no effect on TN-GnRH3 number, whereas BPA once again increased TN-GnRH3 number (similar to what was shown in Figure 1B). Expression levels of *kiss1* (Figure 2B) (ANOVA, $P = .025$; Dunnett's test, $P < .05$) and *gnrh3* (Figure 2D) (ANOVA, $P < .001$; Dunnett's test, $P < .05$) were significantly increased by BPS treatment. As with BPA, BPS had no effect on *kiss2*, *kiss2r, fshβ*, and *sv2* gene expression (Supplemental Figure 1). Furthermore, 100 μg/L BPA showed similar effects on reproductive neuroendocrine-related gene expression in Figure 2 as in the earlier experiments shown in Figure 1D, providing a strong measure of reproducibility and validation of the results.

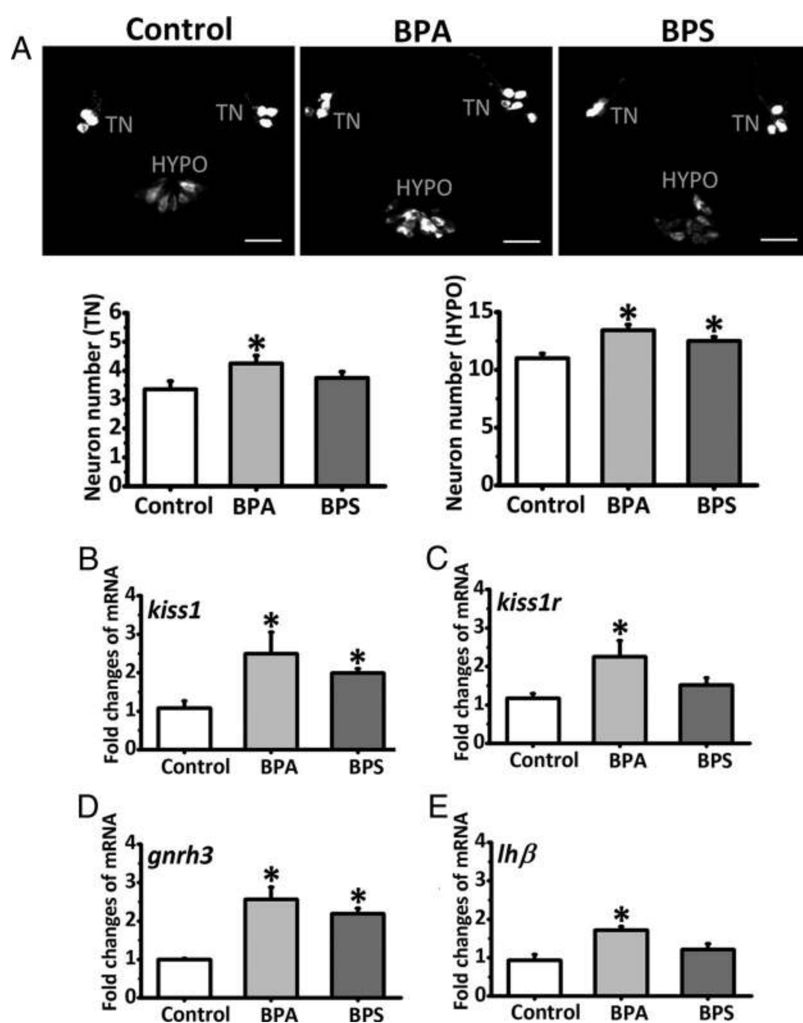

Figure 2. BPS has similar effects as BPA on GnRH3 neuron number and reproductive neuroendocrine-related gene expression at 25 hpf in zebrafish embryos. A, Z-stack of confocal images and summary data of GnRH3 neurons expressed in TN and HYPO after exposure to 100 μg/L BPA and 100 μg/L BPS (n = 14 each). Horizontal bars indicate 20 μm. Effects of BPA (100 μg/L) and BPS (100 μg/L) on expression of reproductive neuroendocrine-related genes are shown for the following: *kiss1* (B), *kiss1r* (C), *gnrh3* (D), and *lhβ* (E) (n = 6 each). Values are shown as mean ± SE. Asterisks indicate significant difference compared with control at $P < .05$ (ANOVA, Dunnett's test).

Role of ERs in mediating effects of BPA and BPS on reproductive neuroendocrine-related gene expression

To determine possible involvement of ERs in mediating the effects of BPA and BPS on the reproductive

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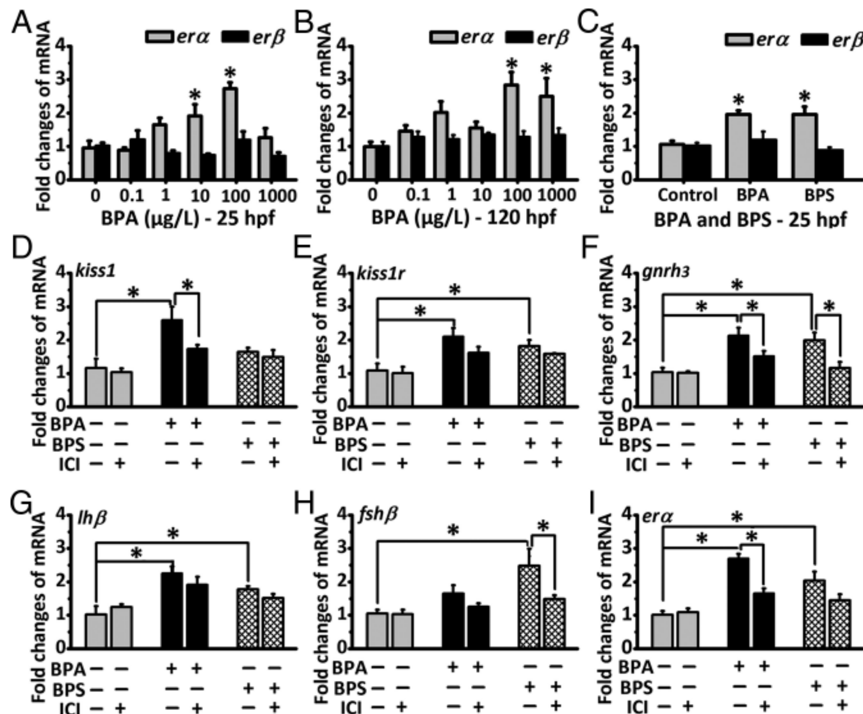

Figure 3. Role of ERs in mediating effects of BPA and BPS on reproductive neuroendocrine-related gene expression in zebrafish embryos. Effects on *er-α* and *erβ* mRNA levels are shown after 25 hpf BPA exposure (A), 120 hpf BPA exposure (B), and 25 hpf BPS exposure (C) (dose of 100 μg/L each). Asterisks indicate significant difference compared with control at $P < .05$ (ANOVA, Duncan's test). Effects on *kiss1* (D), *kiss1r* (E), *gnrh3* (F), *lhβ* (G), *fshβ* (H), and *erα* (I) mRNA levels after exposure to BPA (100 μg/L), BPS (100 μg/L), a mixture of BPA (100 μg/L) and ER antagonist ICI (1 μM), and a mixture of BPS (100 μg/L) and ICI (1 μM) at 120 hpf. Values are shown as mean ± SE (n = 6 each). Asterisks indicate significant differences between treatment groups at $P < .05$ (ANOVA, Duncan's test).

neuroendocrine system, we first analyzed the effects of these endocrine-disrupting chemicals on ER-α (*erα*) and ER-β (*erβ*) gene expression. The mRNA levels of *erα* were significantly increased in response to BPA at 25 (ANOVA, $P < .001$; Dunnett's test, $P < .05$) and 120 hpf (ANOVA, $P = .003$; Dunnett's test, $P < .05$), whereas there was no effect on *erβ* (Figure 3, A–C). As with BPA, treatment with BPS significantly increased the expression of *erα* but not *erβ* at 25 hpf (Figure 3C). These data suggest that ERα, but not ERβ, mediates the effects of BPA and BPS on the reproductive neuroendocrine system of zebrafish. Figure 3 shows that treatment with the ER antagonist ICI significantly inhibited the increase of *kiss1* (Figure 3D), *gnrh3* (Figure 3F), and *erα* (Figure 3I) gene expression in response to 120 hours of treatment of BPA (Duncan's test, $P < .05$). ICI did not significantly inhibit the stimulatory actions of BPS (also 120 h of treatment) on the expression of *kiss1r* (Figure 3E), *lhβ* (Figure 3G), and *erα* (Figure 3I). However, ICI did significantly inhibit the stimulatory action of BPS on expression of *gnrh3* (Figure 3F) and *fshβ* (Figure 3H).

Roles of the THR and AROM B pathways in mediating effects of BPA and BPS on reproductive neuroendocrine-related gene expression

We also investigated two other components of endocrine systems that are known to ultimately impact reproductive functions that are modulated by BPA: the THR and AROM pathways (39, 45–47). We used the THR antagonist AMIO and the AROM inhibitor FAD to determine whether THR and AROM mediate the effects of BPA and/or BPS on reproductive neuroendocrine gene expression (Figure 4). The survival rate of zebrafish embryos was not significantly altered in response to the treatments at 120 hpf (data not shown). AMIO significantly attenuated the stimulatory actions of BPA on gene expression of *kiss1r* (Figure 4B), *gnrh3* (Figure 4C), and *lhβ* (Figure 4D) (ANOVA, $P < .001$; Duncan's test, $P < .05$). Similarly, AMIO significantly attenuated the stimulatory actions of BPS gene expression of *kiss1r* (Figure 4B) and *lhβ* (Figure 4D) but not the other reproductive neuroendocrine-related genes. These findings suggest that THRs partly mediate the stimulatory actions of BPA and BPS on the reproductive neuroendocrine system.

AROM is the key enzyme for local estradiol synthesis from androgen substrate, and is expressed in hypothalamic progenitor cells (18). Figure 4 shows that the AROM inhibitor FAD significantly attenuated the stimulatory actions of BPA on gene expression of *kiss1* (Figure 4A), *gnrh3* (Figure 4C), *lhβ* (Figure 4D), and *erα* (Figure 4F) (ANOVA, $P < .001$; Duncan's test, $P < .05$). Similarly, FAD attenuated the stimulatory actions of BPS on gene expression of *kiss1r* (Figure 4B), *gnrh3* (Figure 4C), *lhβ* (Figure 4D), and *erα* (Figure 4F). These results suggest that AROM enzymatic activity is partly required for mediating the stimulatory actions of BPA and BPS. Together the outcomes from this study provide strong evidence that ERα, THRs, and AROM pathways are all involved in mediating the effects of BPA and BPS on the reproductive neuroendocrine system. The outcomes from pharmacological experiments shown in Figures 3 and 4 are summarized in Table 3. As shown in Table 3, treatment with ICI, AMIO, and FAD significantly inhibited the stimulatory effects of

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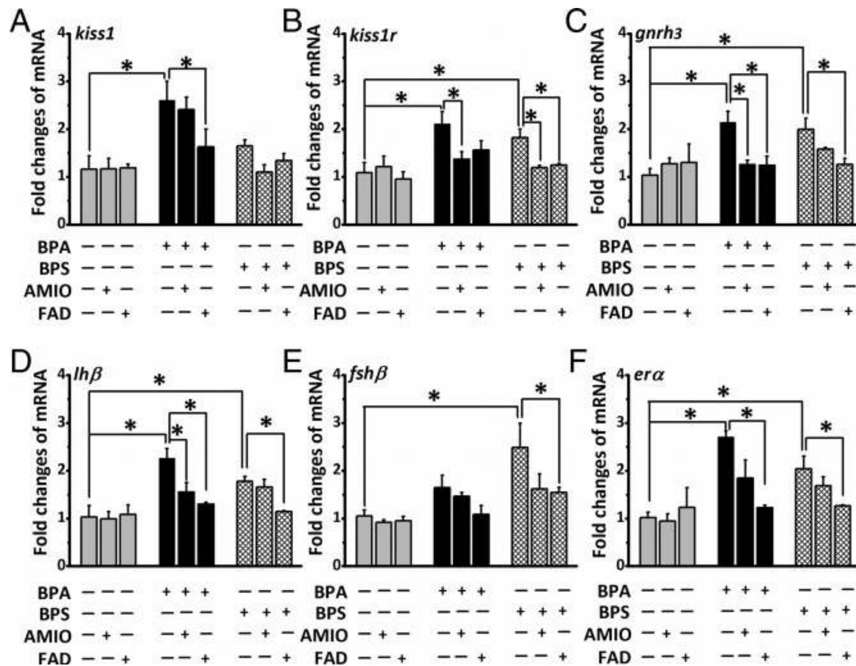

Figure 4. Effects of BPA (100 μg/L) or BPS (100 μg/L) in the presence or absence of the THR antagonist AMIO (1 μM) or the AROM B inhibitor FAD (1 μM) on mRNA levels of the following: *kiss1* (A), *kiss1r* (B), *gnrh3* (C), *lhβ* (D), *fshβ* (E), and *erα* (F). Measurements of mRNA levels were performed at 120 hpf. Values are shown as mean ± SE (n = 6 each). Asterisks indicate significant differences between treatment groups at $P < .05$ (ANOVA, Duncan's test).

BPA and/or BPS on the expression levels of most of the genes investigated. Furthermore, the results show that the expression levels of *erα* were significantly increased by BPA and BPS exposure; these effects were inhibited by the AROM inhibitor FAD, but not the THR antagonist AMIO.

Discussion

Using zebrafish as a model for investigating the impact of endocrine disrupting chemicals on vertebrate embryonic development, we show that ecologically relevant levels of BPA and BPS alter many aspects of the reproductive neuroendocrine system. In response to BPA, hatching time was accelerated. This is very similar to what was seen in an earlier study in embryonic medaka fish (45). Together these results suggest that exposure to low levels of BPA may have a broad implication on embryonic development in teleosts. It was suggested by Herman-Giddens (48) that increased exposure to exogenous hormones was included as one of many potential factors that could advance the timing of puberty. Moreover, continued exposure to BPA beyond the early developmental stage might have an ecological impact on the health and sustainability of fish populations (49). In addition, GnRH3 neuron numbers in TN and HYPO were significantly increased at 25 hpf in response to BPA. This could be due to either BPA having a broad effect on embryonic and/or central nervous system development as suggested by the accelerated rate of hatching or having specific effects on development of certain aspects of the reproductive axis. Given that BPA (and BPS) enhanced expression of some, but not all, of the genes investigated suggests that there is at least some selectivity of these endocrine disruptors' actions on embryonic development.

The expression of reproductive-neuroendocrine related genes such as *kiss1*, *kiss1r*, *gnrh3*, and *lhβ* was significantly increased at both 25 and 120 hpf, whereas *sv2* and *fshβ* were increased only at 120 hpf. Moreover, the alterations of most tested parameters were significantly correlated at both 25 and 120 hpf, indicating a coordinated modulation process in the early development of zebrafish that is sensitive to both dose and duration of varying doses and durations of BPA exposure. Consistent with our present observations, Qin et al (50) have shown that exposure to 15 μg/L BPA increased *gnrh3* and *gnrhr1a* in the brains of adult rare minnow. Vosges et al (51) reported that 17α-ethynylestradiol (EE2), a xenoestrogenic endocrine disrupting chemical, at concentrations as low as 0.1 nM disrupts the ontogeny of the GnRH system by inducing a dose-dependent increase in the number of GnRH3 neurons during zebrafish development. Also, some reproduction-related genes have been found to be up-regulated in response to EE2 in female Coho salmon (52) and in fathead minnow's early-life stages (53). These findings are

Table 3. Role of ER Antagonist ICI, the THR Antagonist AMIO, and the AROM Inhibitor FAD in Mediating Effects of BPA and BPS on Reproductive Neuroendocrine-Related Gene Expression in Zebrafish Embryos

	ICI		AMIO		FAD	
Name	**BPA**	**BPS**	**BPA**	**BPS**	**BPA**	**BPS**
kiss1	S	x	NS	x	S	x
kiss1r	NS	NS	S	S	NS	S
gnrh3	S	S	S	NS	S	S
lhβ	NS	NS	S	NS	S	S
fshβ	x	S	x	NS	x	S
erα	S	NS	NS	NS	S	S

Abbreviations: NS, no significant difference compared with BPA or BPS exposure alone; S, significant inhibition of effects induced by BPA or BPS exposure alone; x, treatment with BPA or BPS alone had no significant effect compared with controls.

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similar to the observations in our study showing the effects of BPA and BPS on GnRH3 neuron number and the expression levels of reproductive neuroendocrine-related genes during early zebrafish development. The earlier studies support our findings that BPA and BPS are acting, at least in part, through an ER-activated pathway.

Our results revealed that BPA had significant effects on hatching rate and early development of the reproductive neuroendocrine system at concentrations as low as 10 μg/L. Environmental concentrations of BPA are known to cause alterations to the reproductive systems in a variety of aquatic vertebrate species. For example, BPA at 2.28 and 22.8 μg/L had sex-altering effects on the amphibian *Xenopus laevis* (54), whereas BPA at 15 μg/L induced *gnrh3* and *gnrhr1a* expression in adult rare minnow (50). In the present study, we found a shift in the sensitivity of reproductive neuroendocrine-related genes as a function of age of the embryo/larvae. During early embryogenesis at 25 hpf, 10 and 100 μg/L of BPA were most effective in up-regulating gene expression but not at a higher dose of 1000 μg/L. This finding is consistent with previous work showing that nonlinear dose responses are commonly observed for hormones and endocrine disrupting chemicals (55). Notably, at 120 hpf, 1000 μg/L was now effective in up-regulating gene expression. We interpret these findings to indicate that the effects of BPA on the expression of reproductive neuroendocrine-related genes depend on the duration of exposure and stage of development. This supports earlier work showing that BPA can have very different effects on development based on the time point of exposure (56).

Our results revealed that BPA and BPS up-regulated the expression of the Kiss1/Kiss1r system, which is widely regarded as an upstream regulator of GnRH neurons, including in fish (57). Furthermore, there was a very high correlation in the present study between *kiss1* and *gnrh3* levels (correlation coefficient of 0.8) in response to BPA. As such, it is possible that endocrine disrupting chemicals' effects on GnRH neuron number and *gnrh* mRNA levels in the present study could have been mediated through activation of the Kiss1/Kiss1r system. Notably, neither BPA nor BPS altered the expression of *kiss2*. Recent work from our laboratory showed that treatment with Kiss1, but not Kiss2, increased the number of TN and HYPO GnRH3 neurons during zebrafish embryogenesis as well as synaptic contacts on TN-GnRH3 neurons (44). Together these findings lend support to the hypothesis that Kiss1 is the primary regulator of the hypothalamo-pituitary axis in zebrafish under normal conditions. However, recent work in zebrafish using a gene knockout strategy showed that expression of *kiss1*, *kiss2, kiss1r*, and *kiss2r* are not required for reproductive functions (58). This suggests that in zebrafish, the Kiss/Kissr systems modulate reproductive functions but are not essential like they are in mammals.

Our results revealed that BPA and BPS affected not only the GnRH3/Kiss1 systems but also *lh*β, *fsh*β, and *sv2* gene expression, which significantly correlated with the changes of *kiss1*, *kiss1r*, and *gnrh3*. FSH and LH, two distinct gonadotropins, form an integral part of the hypothalamo-pituitary-gonad axis and are key players in vertebrate gonadal functions. FSH and LH have been shown to regulate gonad development and reproduction in a similar manner across different species of teleost fishes (59, 60). Earlier work in the hermaphroditic fish *Kryptolebias marmoratus*, showed that BPA exposure increased *fsh*β and *lh*β mRNA levels in brain/pituitary. This is similar to the findings in the present study, in which BPA significantly increased expression of *fsh*β and *lh*β during development, suggesting the potential for BPA exposure to ultimately impact gonadal development and reproduction in fish. SV2 is a component of all vertebrate synapses, including zebrafish, and is important for the efficacy of synaptic communication (61). Therefore, the increase in *sv2* expression after BPA exposure for 120 hpf could result in abnormal synaptic transmission.

BPS, considered an important substitute for BPA in industrial applications, is increasingly used for the production of epoxy resins and paper products and as an anticorrosive agent in epoxy glues and a reagent in polymer reactions (14, 62). It has been considered a safer alternative to BPA for the production of BPA-free products. Earlier work showed that at concentrations as high as 0.1–1 mM (concentrations unlikely to be leached from BPS containing products), BPS showed only slight estrogenic activity in a 4-hour, recombinant, two-hybrid yeast test system (16, 17). In related work, Chen et al (18) showed that 40 μM BPS had 15-fold lower genomic estrogenic activity than BPA. Notably, our results showed that both BPA and BPS are acting through multiple cellular pathways (including THR), not limited to an estrogenic pathway. Consistent with our results, Kinch et al (23) showed that low-dose BPS (1.5 μg/L) resulted in 240% increase in neuronal birth (neurogenesis) within the hypothalamus, in a similar manner as with exposure to BPA. Also, observations by Ji et al (22) showed that exposure of zebrafish to low level BPS (from 0.5–50 μg/L) could affect the feedback-regulatory circuits of the hypothalamo-pituitary-gonadal axis and impair the development of offspring. Therefore, BPA-free plastic products are not necessarily safer than products containing BPA.

Most of the xenoestrogenic endocrine-disrupting chemicals, including BPA, interfere with the normal estrogen signaling pathway by interacting with ERα and/or

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ERβ (63, 64). Our results showed that both BPA and BPS increased expression of *erα* but not *erβ*. This is consistent with recent work showing that BPA increased expression of *erα* but not *erβ* in macrophages of red carp (38). In the present study, treatment with the ER antagonist ICI significantly inhibited the increase of reproductive neuroendocrine-related gene expression in response to 120 hours treatment with BPA and BPS. Together these findings suggest that both BPA and BPS are acting, at least in part, through an ERα-mediated pathway. This is supported by work in mammals showing that ER signaling plays a pivotal role in reproductive neuroendocrine responses to BPA exposure (65, 66).

There is evidence that in addition to affecting ER pathways, BPA also influences THR (67) and AROM pathways (68). Thyroid hormone of maternal origin has been shown to regulate gene expression during early development of the fetal brain, affecting proteins involved in fate specification (69). In zebrafish, thyroid hormones play an important role in development during the embryonic to larval transitory phase (70). Zoeller and coworkers (67, 71) suggested that BPA acts as a TH antagonist in the developing rat brain and mediates the negative feedback effect of TH on the pituitary gland. Also, earlier work in medaka fish demonstrated that BPA-induced acceleration of embryonic development and hatching time were blocked by the THR antagonist AMIO, suggesting an important role of the THR pathway in mediating the effects of BPA on development (46). This finding is consistent with our observations in developing zebrafish that AMIO significantly attenuated the stimulatory actions of BPA and BPS on reproductive neuroendocrine-related gene expression. The present findings reveal that THRs partly mediate the stimulatory actions of BPA and BPS on the reproductive neuroendocrine system.

Cytochrome P450 aromatase is central to estrogen synthesis, catalyzing the final, rate-limiting step in the conversion of androgens to estrogens. Previous work showed that brain AROM is activated in response to exposure to estrogenic chemicals including E2, EE2, and BPA in the adult male zebrafish (72). In embryonic zebrafish, Kinch et al (23) showed that low-dose (1.5 μg/L) BPA and BPS exposure induced precocious hypothalamic neurogenesis that was mediated by AROM signaling. Moreover, our present results showed that the AROM inhibitor FAD significantly attenuated the stimulatory actions of BPA and BPS on reproductive neuroendocrine-related gene expression. This outcome revealed that AROM enzymatic activity is also partly required for mediating the stimulatory actions of BPA and BPS on the reproductive neuroendocrine system.

In mammal and teleost fish models, the functional interaction between ER-, THR-, and AROM-activated pathways can serve as an important regulatory link in the endocrine systems (73, 74). It is well known that E2 upregulates AROM gene expression and that these effects can be blocked by an ER antagonist, suggesting that ERs are involved in the E2-dependent induction of aromatase activity (40, 75, 76). In addition, the present results show that expression levels of *erα* were significantly increased by BPA and BPS exposure, and these effects were significantly attenuated by the AROM inhibitor FAD, suggesting that the interaction between ER and AROM signaling pathways was bidirectional in zebrafish in response to endocrine-disrupting chemicals. However, we have not observed a similar interaction between the ER and THR signaling pathways. The present study provides foundational information using a unique model system for investigating mechanisms by which endocrine disrupting chemicals interfere with early-life development. Moreover, this is the first study to describe the impact of low-level BPA and BPS exposure on the Kiss/Kissr system during embryonic development in any species. It also provides important supporting evidence that BPS is not necessarily a safer alternative to BPA, as suggested by earlier studies focusing solely on its estrogenic activities.

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