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Full Length Article

Bisphenol F causes disruption of gonadotropin-releasing hormone neural development in zebrafish via an estrogenic mechanism

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ABSTRACT

Gonadotropin releasing hormone (GnRH) neurons in the brain are the main controllers of reproduction and reproductive behavior in most vertebrates, and are susceptible to endocrine disruption by different bisphenols. While the endocrine disrupting properties of bisphenol A have been well documented, commonly used analogues such as bisphenol F (BPF) are not as well studied. In this study we examined the effects of early, low-dose, chronic BPF exposure on the development of the GnRH neural system in the zebrafish embryo.

Using a transgenic zebrafish model system with GnRH3 neurons tagged with green fluorescent protein (GFP), developing GnRH neurons in both the terminal nerve (TN) and preoptic area (POA) were observed. These are neuronal populations with the former associated with allied reproductive behaviors and the latter associated with pituitary-gonadal axis control. Embryos were exposed *in vitro* to 0.25, 0.5 and 1 μM BPF from fertilization to 3 days post fertilization (dpf). At 0.25 μM BPF exposure, both POA- and TN- GnRH3 neurons showed significant reductions in neural area at 2 dpf that did not persist to 3 dpf. The higher BPF doses did not show neuron size differences at 2 dpf, but showed reduction in TN-GnRH3 neuron area at 3 dpf. These effects of BPF were closely mimicked by different doses of estradiol. An estrogen antagonist, ICI, mitigated BPF effects on the embryo. This is the first study to show that BPF affects the developing GnRH neural system via an estrogen-mediated pathway.

1. Introduction

Bisphenols are widely used synthetic compounds present in substances such as polycarbonate plastics, epoxy resins, paper and food packaging (Cantonwine et al., 2010; Andrianou et al., 2016; Moreman et al., 2017). Over the last decade the endocrine disrupting property of bisphenol A (BPA) has been extensively studied (Rochester, 2013; Vandenberg et al., 2013; Rezg et al., 2014; Ng et al., 2015; Inagaki et al., 2016a), especially with regard to developmental effects and fetal exposure (Schoenfelder et al., 2002; Kabuto et al., 2004; Lee et al., 2008; Brieño-Enríquez et al., 2012; Edlow et al., 2012; Snijder et al., 2013). Given overwhelming evidence of fetal risk, many countries are restricting the use of BPA in products including infant feeding bottles (Quitmeyer and Roberts, 2007; FDA, 2016), paper (Liao and Kannan, 2013) and food packaging (Baluka and Rumbeiha, 2016). Public concern and governmental restrictions are causing manufacturers to develop "BPA-free" products (Cano-Nicolau et al., 2016). However, in many of these there is a prevalent use of BPA analogues such as bisphenol F, S and AF (BPF, BPS, BPAF) (Lee et al., 2015; Rochester and Bolden, 2015; Moreman et al., 2017). These analogues have markedly less research on their effects on animal and environmental health (Rochester and Bolden, 2015).

BPF is used for making epoxy resins and coatings, lacquers, plastics, water pipes, dental sealants and food packaging (Liao and Kannan, 2013; Lee et al., 2015; Rochester and Bolden, 2015; Andrianou et al., 2016). Measurable concentrations of BPF are found in a wide range of products including toothpaste and food (Liao and Kannan, 2013, 2014; Lee et al., 2015). In humans, BPF is detected in concentrations up to 212 ng/ml in urine samples of non-occupationally exposed Americans (Liao et al., 2012a; Rochester and Bolden, 2015). Environmental studies performed by Chen and colleagues (Chen et al., 2016) also showed BPF is detectable in sediment (1.44 ng/g (U.S); 3.57 ng/g (Japan)), water (215 ng/L (Japan); 277 ng/L (China)) and in food (0.93 ng/g (U.S)).

Exposure to 1 μM BPF significantly activated the expression of the estrogen receptor marker in the developing zebrafish brain (Cano-Nicolau et al., 2016) and increased thyroid hormone levels in 6 day old zebrafish (Huang et al., 2016). At 1 mg/L BPF impaired the reproductive function of zebrafish and also increased estradiol levels (Yang et al., 2017). Evidence suggests that BPF exposure in the environment interferes with normal embryonic development and exhibits estrogenic effects similar to BPA (Chen et al., 2016). *in vivo* studies suggest that BPF is estrogenic, androgenic, and thyroidogenic

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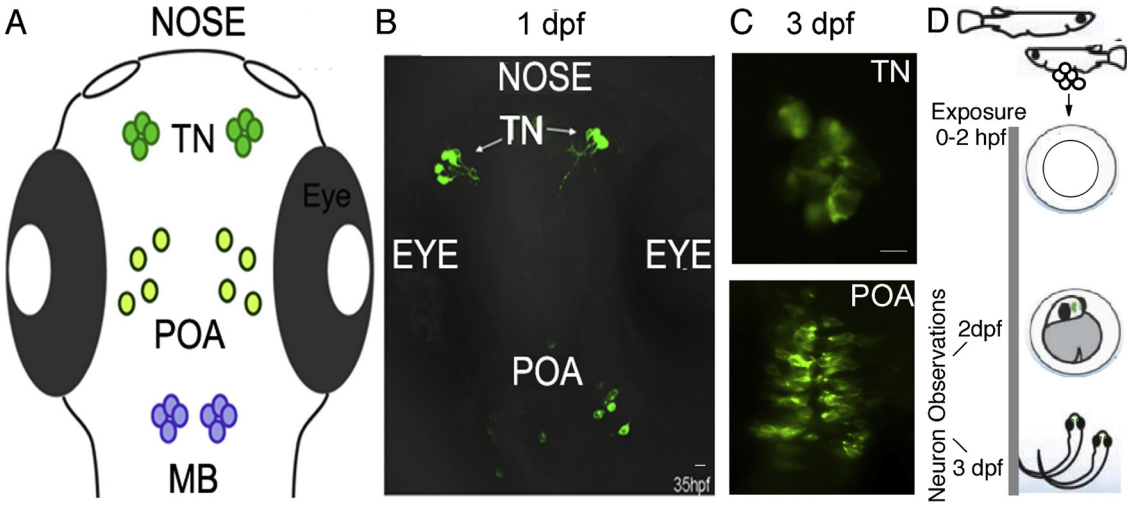

Fig. 1. Schematic and representative images of GnRH3-GFP neurons in the transgenic zebrafish embryo and experimental timeline. A) Schematic of different neuron populations in zebrafish embryo. B) View of whole head of a 1 dpf embryo showing TN and POA GnRH3-GFP populations alongside anatomical landmarks. C) Closer look at the GnRH3 neuron populations at 3 dpf, representative of images used for measurements in this study. D) Timeline of treatment exposure, with fertilized embryos exposed to different chemicals starting 0–2 hpf. Chronic exposure until 3dpf, with neuron observations at 2 and 3 dpf. Scale bar is 10 μm. TN- terminal nerve; POA- preoptic area; MB-midbrain; dpf – days post fertilization.

(Higashihara et al., 2007) and *in vitro* studies showed cytotoxicity and cellular dysfunction (Rochester and Bolden, 2015). Thus there is accumulating evidence of the endocrine disrupting nature of BPF potentially through an estrogen-mediated pathway.

Gonadotropin releasing hormone neurons (GnRH) in the brain are the main controllers of reproduction and allied behaviors in most vertebrates (Abraham et al., 2009). There are three types of GnRH neurons in the vertebrate brain: GnRH1 in the preoptic area (POA-GnRH) controlling the pituitary-gonadal functions; GnRH2 in the midbrain, associated with metabolic regulation and GnRH3 neurons in the terminal nerve (TN-GnRH) with links to allied reproductive behaviors (Millar, 2005; Kawai et al., 2009; Oka, 2009; Zohar et al., 2010; Roa, 2013). In zebrafish both the POA and TN express GnRH3 (Fig. 1) (Ramakrishnan et al., 2010).

Given the estrogenic nature of endocrine disruption by bisphenols, different studies have examined their effects on the GnRH reproductive neuroendocrine system. Bisphenols, such as BPA, BPS, alter the GnRH neural system in the teleost embryo with increased GnRH mRNA expression (Lee et al., 2012), increased GnRH fluorescence (Lee et al., 2012; Inagaki et al., 2016a, 2016b) and increased GnRH neuron numbers (Qiu et al., 2016). Embryonic BPA exposure was also shown to reduce GnRH neuron area in larvae (Inagaki et al., 2016a, 2016b). Chronic, low dose bisphenol exposure resulted in altered reproductive end points in teleost fish including advanced sexual maturation (Ramakrishnan and Wayne, 2008; Yang et al., 2017), altered hatch rates (Ramakrishnan and Wayne, 2008; Lee et al., 2012; Inagaki et al., 2016a) and increased vitellogenin in males (Le Fol et al., 2017). In some studies, bisphenol effects on GnRH neurons persisted across generations (Inagaki et al., 2016b).

In this study we examined the effects of low-dose, chronic exposure to BPF on developing GnRH3 neuron populations in the zebrafish embryo. Transgenic zebrafish with GnRH3 neurons tagged with green fluorescent protein (GFP) were used to observe both POA and TN-GnRH3 neuron populations at 2 and 3 days post fertilization (dpf). Embryos were exposed to 0.25, 0.5 and 1 μM BPF from fertilization through hatch and GnRH neuron areas were measured. Putative estrogenic effects of BPF were determined by treating embryos to different doses of β-estradiol (0.2, 1 and 5 nM) and concomitant exposure of embryos with an estrogen antagonist (1 μM ICI) in the presence of BPF. While there have been numerous studies examining BPA effects on development, specifically in zebrafish, there is some evidence indicating that BPA analogues could have different effects at varying concentrations and potentially act via different pathways (Rochester and Bolden, 2015). This is the first study to show that low-dose, chronic BPF exposure alters the developing GnRH neural system in the zebrafish embryo via an estrogenic pathway.

2. Materials and methods

2.1. Animals and housing

Transgenic zebrafish (*Danio rerio*) embryos and larvae with GFP tagged GnRH3 neurons were used in this study. The gene construction and generation of this transgenic line was described previously (Ramakrishnan et al., 2010). Adult zebrafish were maintained in a house-built tank system with flow-through filtered fish water (Kim et al., 2009). The fish were under 10/14-hour dark/light cycle, and were fed twice daily with live brine shrimp and flake food (Tetramin, USA).

The study was designed based on basic principles of experimental design (Fry, 2013), having sufficient biological variability in subjects and using random group assignment and blind analysis for data collection. Breeding groups were selected from amongst 8 adult zebrafish tanks with around 20 zebrafish per tank. Breeding chambers containing one male and two females were set up weekly. Pairs of fish that were bred were rotated. Embryos from different breeding pairs were pooled to randomize the eggs. Eggs were collected after fertilization and kept in standard petri-dishes containing 20 mL of embryo medium (EM) and placed in an incubator maintained at 28 °C. Procedures were performed in accordance with the mission of the Institutional Animal Care and Use Committee at the University of Puget Sound in Tacoma, WA.

2.2. Reagents and solutions

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted. EM was prepared using a 50x EM stock solution (250 mM NaCl; 8.6 mM KCl; 16.5 mM $CaCl_2 \cdot 2\ H_2O$; 16.5 mM $MgSO_4 \cdot 7\ H_2O$); 20 mL stock EM was diluted with 980 mL distilled water (dH_2O), and 25 μl aqueous methylene blue to reach a pH between 7.2 and 7.4. Fish water contained: 0.187 g of Instant Ocean (United Pet Group, OH); 8.6 mg $CaSO_4$; 12.6 mg $NaHCO_3$ in 1 L dH_2O. 2% paraformaldehyde was used as a fixative. Ethyl 3-aminobenzoate

Table 1
GnRH3-GFP neuron areas under different treatment conditions. Area in μm^2 is expressed as Mean ± Standard Error of Mean (SEM). Two different populations of GnRH3 neurons – in the TN (above) and the POA (below) are indicated. Neuron size was measured at both 2 and 3 days post fertilization (dpf). Embryos were exposed to different bisphenol F (BPF) concentrations of 0.25, 0.5 and 1 μM; β-estradiol – 0.2, 1 and 5 nM; estrogen antagonist ICI alone at 1 μM, and concomitant BPF + ICI exposures. * indicates significant differences from controls ($p < 0.05$). Differences across rows (2dpf vs. 3dpf) was determined using Students *t*-test. $ indicates significant differences between 2dpf and 3dpf embryos, $p < 0.05$.

	TREATMENTS									
	Control	BPF			β-estradiol			ICI		
		0.25μM	0.5μM	1μM	0.2 nM	1 nM	5nM	1μM ICI	0.25μM BPF +1uM ICI	0.50μM BPF +1uM ICI
TERMINAL NERVE (TN)										
2dpf	31.26 ± 1.59	**18.39 ± 2.53** *,$	28.83 ± 2.86	30.02 ± 1.53	25.14 ± 1.58 $	30.40 ± 3.34	32.02 ± 1.43	28.82 ± 1.08 $	27.81 ± 0.96 $	32.34 ± 2.26
3dpf	36.59 ± 2.28	32.87 ± 0.82	**31.53 ± 0.88** *	**30.87 ± 0.72** *	35.15 ± 2.24	**27.48 ± 2.11** *	**27.21 ± 1.59** *	31.90 ± 1.00	33.59 ± 1.72	34.08 ± 1.37
PREOPTIC AREA (POA)										
2dpf	31.44 ± 2.02	**14.52 ± 1.22** *,$	30.21 ± 1.83	28.22 ± 1.60 $	26.38 ± 0.55 $	33.90 ± 1.82 $	33.32 ± 1.46	29.66 ± 1.41	28.10 ± 0.80	30.36 ± 1.56
3dpf	31.08 ± 1.84	32.27 ± 0.74	34.40 ± 2.37	33.10 ± 1.35	31.39 ± 2.20	27.14 ± 2.61	31.23 ± 5.61	31.39 ± 1.63	30.14 ± 1.26	30.58 ± 0.75

methanesulfonate salt (MS-222) was used as an anesthetic (4 mg in 10 mL dH_2O; Fluka Analytical). Stock solutions were prepared in 100% ethanol: 8 mg/mL BPF; 1 mg/mL β-estradiol and 1 mM ICI 182,780 (ICI, Tocris biosciences; Minneapolis, MN).

2.3. Experimental design and timeline of exposure

In previous BPF experiments where zebrafish embryos and larvae were exposed to BPF via the embryo medium, the BPF detected in exposure solutions (with renewal) at 5 dpf was in the same concentration range to initial BPF exposure levels (Huang et al., 2016). We followed similar embryo-medium borne BPF exposure in our experiments. Eggs were randomly assigned to different treatment and control groups. Exposure to different treatments started between 0–2 hours post fertilization (hpf) through 3 dpf. Neuron observations were performed on embryos at 2 and 3 dpf. 10% of solution in the petri dish was replaced daily with either treatment or control EM. Stocks were dissolved in EM to make final treatment solutions at the following concentrations: BPF – 0.25 μM, 0.5 μM and 1 μM; β-estradiol – 0.25 nM, 0.5 nM and 1 nM; and ICI 1 μM. In mechanistic experiments, embryos were exposed to either 1 μM ICI alone or 1 μM ICI in conjunction with 0.25 μM or 0.5 μM BPF. Only these BPF concentrations were used for mechanistic experiments, as they elicited divergent results on GnRH3 neuron sizes. The percent ethanol in final solutions was equal to or less than 0.0025%. Control solution had equal amounts of ethanol vehicle in EM.

2.4. Imaging and analysis

Embryos (at both 2 and 3dpf) were placed in MS-222 and manually hatched and fixed in 2% paraformaldehyde (in 0.02 M PBS) for two hours at room temperature or overnight at 4 °C. Embryos were then washed 3 x 10 min in PBS and stored in 100% acetone at −20 °C until ready for imaging. Embryos were transected and their heads were placed ventral side up on slides and mounted with DPX slide mount. GnRH3-GFP neuron populations were imaged using epifluorescence under an inverted Nikon D-Eclipse microscope and the Nikon Instruments Systems Elements software (Nikon Instruments Inc.). GnRH3-GFP neuronal populations were visualized with 488 nm excitation and 512 nm emission filters. GnRH3-GFP neuron sizes were determined in both 2 and 3 dpf embryos in both the POA and TN populations (Fig. 1). Fluorescence images under 20x objectives were taken for each fish. All images were then analyzed using ImageJ software (National Institute of Health).

In the embryo the TN-GnRH3 population contains a cluster of 5–10 neurons on each side of the brain. The numbers of TN-GnRH3 neurons were counted. There are many more POA neurons at different depths (Fig. 1). It was not possible to accurately count POA neuron numbers, as there are many at different depths. For each fish, the area of individual GnRH3-GFP neurons was measured and the average area of neurons in each population was calculated at both 2 and 3 dpf. Averages of these neuron areas for each population were then determined for each treatment group and reported as Mean area ± standard error of mean (SEM) in μm^2, with the number of fish per treatment group (n). Results from TN- and POA-GnRH3 neurons were analyzed separately. Statistical analysis was performed using VassarStats software (www.vassarstats.net). A two-way ANOVA (treatment x dpf) was conducted to test differences in treatment group across two different age groups. If a significant main or interaction effects was found the data were further examined using a one-way ANOVA for dose effects on specific days, followed by Tukey HSD post hoc test. $P < 0.05$ was considered significant. For row effects, data between 2dpf and 3dpf embryos was compared in each treatment group using Students *t*-test. $P < 0.05$ was considered significant. All image analysis and measurements were conducted blind to treatment group. Values two standard deviations away from the mean were considered outliers and excluded.

3. Results

3.1. Bisphenol F treatment alters GnRH neuron size in embryos

To determine if chronic, low dose BPF treatment affects development of GnRH neurons, we examined GnRH3 neuronal area in both the TN and POA in 2 and 3 dpf embryos (Table 1, Fig. 2). Two way ANOVA of TN neurons showed significance in both main effects of both dpf ($F_{1,221} = 28.79$, $p < 0.0001$) and treatment ($F_{3,221} = 4.9$, $p = 0.0026$), as well as interaction ($F_{3,221} = 12.57$, $p < 0.0001$). Two way ANOVA of POA neurons also showed significance in both main effects of both dpf ($F_{1,107} = 45.8$, $p < 0.0001$) and treatment ($F_{3,107} = 18.5$, $p < 0.0001$), as well as interaction ($F_{3,107} = 11.87$, $p < 0.0001$).

At 2 dpf, embryos exposed to 0.25 μM BPF showed a significant 41% reduction in the TN-GnRH3 neuron area (Fig. 2A; Mean area ± SEM; n = number of fish: 18.39 ± 2.53, n = 19; $F_{3,74} = 8.81$; $p < 0.0001$; Tukey HSD [0.01] = 9.6) and a significant 53% decrease in POA-GnRH3 sizes (Fig. 2B; 14.52 ± 1.22, n = 15; $F_{3,50} = 27.05$; $p < 0.0001$; Tukey HSD [0.01] = 7.37) when compared to the vehicle-

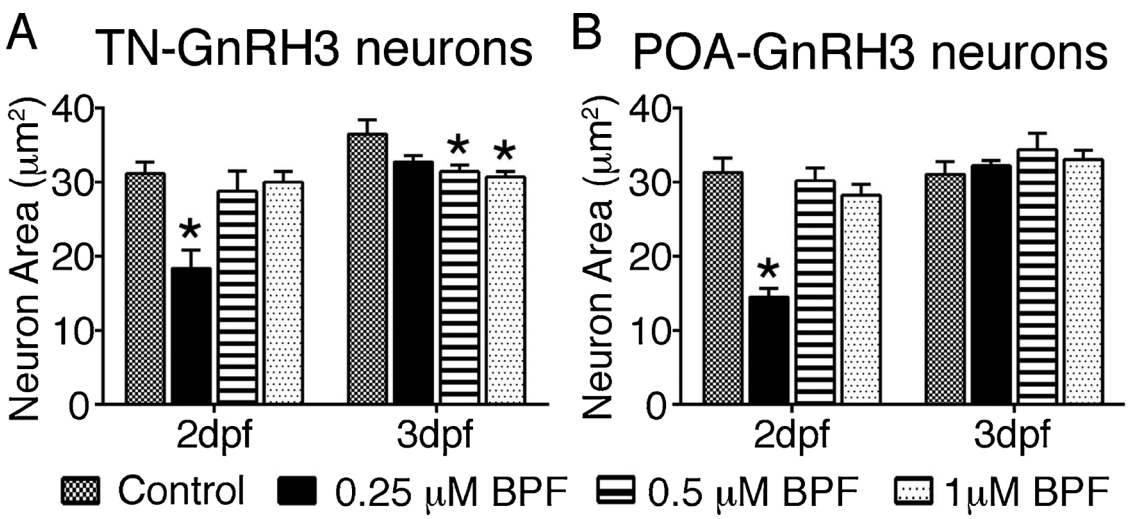

Fig. 2. BPF alters GnRH3-GFP neuron sizes. BPF effects on GnRH neuron area for both the A) TN and B) POA are shown. Data indicated as Mean area in μm^2. Error bars show standard error of mean. Control embryos were exposed to the vehicle ethanol in the embryo medium. Three different BPF doses of 0.25, 0.5 and 1 μM BPF were used. * indicates $p < 0.05$; ANOVA followed by posthoc Tukey HSD was used to determine significance. dpf – days post fertilization, TN- terminal Nerve; POA – preoptic area.

treated controls (TN: 31.25 ± 1.59, n = 16; POA: 31.44 ± 2.02, n = 10).

TN-GnRH3 neuron area at 3 dpf (Fig. 2A) showed a significant 13% decrease with 0.5 μM BPF (31.53 ± 0.88; n = 32) and a significant 15% decrease with 1 μM BPF (30.88 ± 0.72; n = 50) exposure when compared to control embryos (36.59 ± 2.28; n = 16; $F_{3,146} = 4.84$; $p < 0.005$; Tukey HSD [0.01] = 4.59). However the significant reduction observed with the 0.25 μM treatment at 2 dpf was no longer observed at 3 dpf (Table 1, Fig. 2A). At 3 dpf, there were no significant differences in the POA-GnRH3 neurons across treatment groups ($F_{3,56} = 1.07$; $p = 0.37$; Fig. 2B; Table 1).

Comparison of 2 and 3dpf GnRH neuron sizes using Student's *t*-test showed no differences in either the TN ($p = 0.05$) or POA ($p = 0.86$) groups in the control embryos. Under 0.5 μM BPF both the TN and POA neurons at 2dpf were significantly smaller than the 3dpf neurons. While no differences were observed with the 0.5 μM BPF treatment, POA neurons under 1 μM BPF at 2dpf were significantly smaller than 3dpf neurons (Table 1). No differences were seen in TN-GnRH3 neuron numbers at either 2 or 3 dpf under all three doses of BPF (Average number of Neurons ± SEM (n = number of fish) – 2 dpf embryos Control: 4.91 ± 0.6 (n = 6); 0.25 μM BPF: 4.4 ± 0.7 (n = 5); 0.5 μM BPF: 3.8 ± 0.4 (n = 5); 1 μM BPF 6.14 ± 0.8 (n = 7), 3 dpf embryos Control: 6 ± 0.4 (n = 6); 0.25 μM BPF: 4.8 ± 0.4 (n = 5); 0.5 μM BPF: 4.75 ± 0.5 (n = 4); 1 μM BPF 6.4 ± 0.5 (n = 5)).

3.2. Estrogen agonist effects on GnRH neurons

To elucidate the mechanism behind BPF action, embryos were dosed with β-estradiol at 0.2, 1 and 5 nM concentrations from fertilization through 3 dpf (Table 1; Fig. 3). Two way ANOVA (dpf x treatment) of TN-GnRH neurons exposed to estrogen agonist, showed significant main effect of treatment ($F_{3,111} = 4.05$, $p = 0.0091$) as well as interaction ($F_{3,111} = 5.54$, $p = 0.0014$) but not of dpf ($F_{1,111} = 1$, $p = 0.3196$). Two way ANOVA (dpf x treatment) of POA-GnRH neurons exposed to estrogen agonist, showed significant effect of interaction ($F_{3,77} = 3.11$, $p = 0.0318$) but not main effects of dpf ($F_{1,77} = 0.38$, $p = 0.54$) or treatment ($F_{3,77} = 2.12$, $p = 0.1054$).

At 2 dpf there was a 19% reduction in TN-GnRH3 neuron size when embryos were treated with 0.2 nM β-estradiol (25.13 ± 1.58, n = 11) compared with controls. This was however, not significant ($F_{3,45} = 2.42$, $p = 0.08$). There was no size difference of neurons treated with 1 nM (30.40 ± 3.34; n = 10), or 5 nM β-estradiol (32.02 ± 1.43; n = 9) (Fig. 3A). At 2 dpf there was a 16% reduction in POA-GnRH3 neuron size when embryos were treated with 0.2 nM β-estradiol (25.13 ± 1.58; n = 11) when compared to control neurons (31.44 ± 2.02; n = 10). These reductions were not significant. POA-GnRH3-GFP neuron size at 2 dpf at both 1 nM (33.90 ± 1.82; n = 12), and 5 nM β-estradiol (33.32 ± 1.46; n = 9) were not different than the controls (Table 1; Fig. 3B).

At 3 dpf there were no significant changes in TN-GnRH3 neuron size after treatment with 0.2 nM β-estradiol (35.15 ± 2.24; n = 10), but there was a significant 24% decrease in TN-GnRH3 neuron size after treatment with 1 nM β-estradiol (27.48 ± 2.11; n = 12) and a 25% decrease with 5 nM β-estradiol (27.21 ± 1.58; n = 20) treatment when compared to the control (Fig. 3A; 36.59 ± 2.28, n = 16; $F_{3,65} = 6.75$; $p < 0.00052$; Tukey HSD [0.05] = 7.54). β-estradiol treatment had no effect on POA-GnRH3-GFP neurons at 3 dpf ($F_{3,36} = 0.75$; $p = 0.53$; Table 1; Fig. 3B).

With 0.2 nM β-estradiol treatment both TN and POA neurons at 2dpf were smaller than those at 3dpf (Table 1). These differences were not noted with 5 nM β-estradiol. The POA-neurons at 1 nM β-estradiol showed a significant reduction between 2 and 3 dpf.

3.3. Estrogen antagonist ICI blocks BPF effects on GnRH neuron size

Given that β-estradiol mimicked some BPF effects, we examined if an estrogen antagonist, ICI, (Qiu et al., 2016) would block these observed effects. As exposure to 1 μM BPF concentration had similar effects to 0.5 μM BPF on the GnRH populations, only the lower BPF concentrations (0.25 and 0.5 μM) were examined. Embryos were treated with 1 μM ICI alone or 1 μM ICI in conjunction with 0.25 μM or 0.5 μM BPF. Two way ANOVA of ICI exposed TN neurons showed significance in both main effects of both dpf ($F_{1,146} = 18.38$, $p < 0.0001$) and treatment ($F_{3,146} = 3.69$, $p = 0.0135$), but not interaction ($F_{3,146} = 0$, $p = 1$). Two way ANOVA of POA neurons showed no significance with either main effects (dpf - $F_{1,117} = 2.52$, $p < 0.0001$; treatment ($F_{3,117} = 1.73$, $p = 0.165$), or interaction ($F_{3,117} = 0$, $p = 1$).

At 2 and 3 dpf, ICI alone did not have any significant effects on TN (Fig. 4A) or POA-GnRH3 (Fig. 4B) neuron size (Table 1). Similarly following concomitant BPF/ICI treatments at any dose, neuron size did not differ from controls in the TN (Fig. 4A) or POA (Fig. 4B) population at both 2 and 3 dpf (0.25 μM BPF + 1 μM ICI: 27.81 ± 0.96; n = 26; 0.5 μM BPF + 1 μM ICI: 32.34 ± 2.25; n = 12). 2dpf TN-GnRH neuron sizes were significantly smaller than those of 3dpf both for ICI

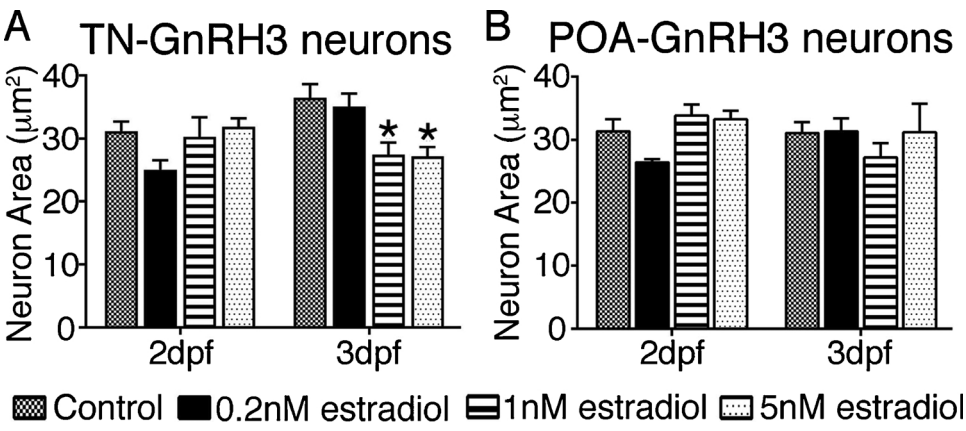

Fig. 3. β-estradiol effects on GnRH3-GFP neurons. Estradiol effects on GnRH neuron area for both the A) TN and B) POA are shown. Data indicated as Mean area in μm^2. Error bars show standard error of mean. Control embryos were exposed to the vehicle ethanol in the embryo medium. Three different estradiol doses of 0.2, 1.0 and 5 nM estradiol were used. * indicates $p < 0.05$; ANOVA followed by posthoc Tukey HSD was used to determine significance. dpf – days post fertilization, TN- terminal nerve, POA – preoptic area.

treatment alone, as well as for concomitant exposure of ICI with 0.25 μM BPF. This was not observed at the higher BPF/ICI treatment.

4. Discussion

The current study addressed the effects of embryonic exposure to BPF on GnRH3 neuron development in zebrafish. We report that chronic low dose BPF exposure affects early embryonic development of GnRH3 neurons in a dose dependent manner. BPF has different effects on two distinct GnRH populations. We also show that many of these BPF dose effects are replicated by different doses of β-estradiol. Further, ICI, an estrogen antagonist, effectively blocks BPF effects. This is one of the first studies to examine the effects of BPF on the GnRH neural populations in the developing embryo and suggests it acts via an estrogenic pathway.

4.1. Low dose, chronic exposure to Bisphenol F affects the developing embryo

The U.S. EPA considers "low dose" effects to be those reported from studies that use doses lower that those used in traditional toxicologic studies. For BPA the lowest observed adverse effect level is 50 mg/kg/day for vertebrates (vom Saal and Hughes, 2005). In aquatic organisms the low dose cut off was set to 17.2 mg/L (Vandenberg et al., 2013). However, research supports the idea that chronic exposure to concentrations well below this standard presents a risk to aquatic species by targetting neurological and neuroendocrine systems. BPF is becoming more prevalent and has been found in food, dust, sediment, receipts, and sewage sludge (Lee et al., 2015; Yamazaki et al., 2015; Yu et al., 2015; Andrianou et al., 2016; Thayer et al., 2016). While some studies have reported levels of 212 ng/mL (1 μM) in urine of non-occupationally exposed humans (Liao et al., 2012b; Rochester and Bolden, 2015), others indicate lower levels around 0.54 ng/mL (Ye et al., 2015).

The concentrations of BPF used in this study (1 μM, 0.5 μM and 0.25 μM) are both environmentally relevant and are similar to or lower than concentrations in comparable experiments with bisphenol analogues that have shown effects on sexual maturation (Ramakrishnan and Wayne, 2008) and rates of hatch (Ramakrishnan and Wayne, 2008; Lee et al., 2012; Inagaki et al., 2016a). In rats low dose BPF exposure induced uterine growth in immature rats, decreased body weight, decreased T3 levels, increased T4 levels, and increases testes, liver, thyroid, and brain weight (Higashihara et al., 2007; Rochester and Bolden, 2015). In cell cultures, BPF at concentrations greater than 30 μM affects cell viability and increased production of hormones including estrogen and progesterone while decreasing cortisol (Feng et al., 2016). Zebrafish embryos exposed until 6 dpf to BPF concentrations in the range of that we used here (0.1–1 μM) showed elevated thyroid stimulating hormone levels, as well as altered gene transcription in hypothalamic-pituitary-thyroid (HPT) axis (Huang et al., 2016). In the developing zebrafish brain exposure to 1 μM BPF stimulated the expression of the estrogen receptor markers and cyp19a1b gene expression (coding for aromatase) (Cano-Nicolau et al., 2016). BPF showed binding affinity to ERα receptors, albeit at a 5 fold lower affinity than BPA (Cano-Nicolau et al., 2016). Thus even at these low doses, similar to concentrations used in this study, BPF affects multiple hormonal systems in the developing embryo.

4.2. BPF alters size of GnRH neurons

Here we show that BPF induced changes in size of GnRH3 neurons

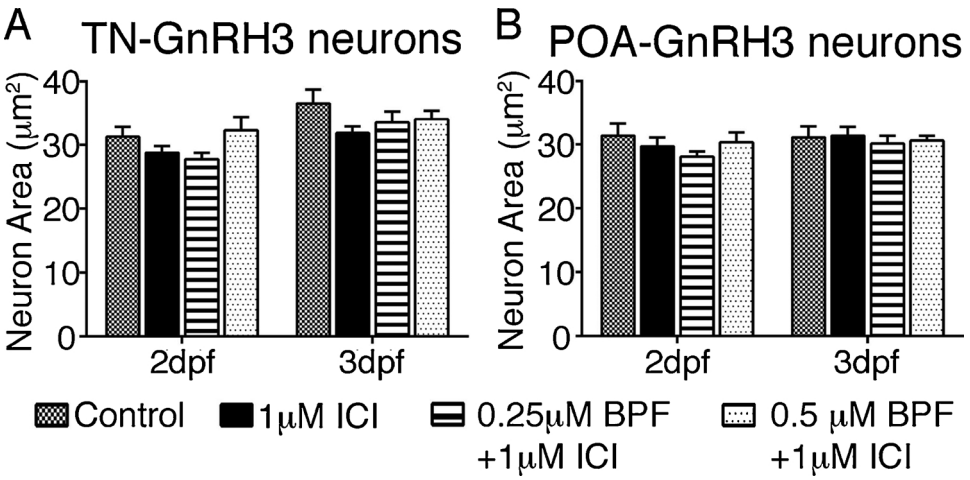

Fig. 4. ICI mitigates effects of BPF on GnRH3 neurons. Effects on GnRH neuron area at both 2 and 3 dpf for both the A) TN and B) POA are shown. Data indicated as Mean area in μm^2. Error bars show standard error of mean. Control embryos were exposed to the vehicle ethanol in the embryo medium. Columns indicate different treatments of control alone, ICI alone, ICI with 0.25 and 0.5 μM BPF. No significance was found across treatment groups in either population. dpf – days post fertilization, TN- terminal nerve, POA – preoptic area.

in the developing embryo. Neuroendocrine systems in mammals as well as fish have been shown to have changes in cell size under different conditions (Olivereau and Olivereau, 1990; Stern and Armstrong, 1998; Hofmann and Fernald, 2000). Size of GnRH neurons has been linked to altered synthesis of GnRH (Bushnik and Fernald, 1995; White et al., 2002; Davis and Fernald, 2004). GnRH neurons show size-related changes to electrical properties such as altered action potential duration, interspike interval and firing rate, which are correlated with efficacy of hormonal release (Dutton and Dyball, 1979; Bicknell, 1988; Yamashita et al., 2002; Greenwood and Fernald, 2004; Leng et al., 2010). Cell input resistance and membrane capacitance are also altered by GnRH neuron size (Greenwood and Fernald, 2004). GnRH neurons have also been shown to have somatic exocytotic release of hormones, which depends on neural size (Ishizaki et al., 2004; Abe and Oka, 2006, 2011; Kawai et al., 2009; Oka, 2010). The reduction in GnRH3 neuron sizes observed in our study could affect GnRH neural activity and hormonal release.

BPA has been shown to lower GnRH induced pituitary release and disrupt GnRH pulsatility (Fernández et al., 2009). Previous studies have observed bisphenol effects on GnRH neuron numbers in rats (Bai et al., 2011) and fish (Qiu et al., 2016). Bisphenols have also been shown to alter GnRH mRNA levels (Lee et al., 2012) and GnRH-GFP fluorescence (Lee et al., 2012; Inagaki et al., 2016a,b). While Lee et al. (2012) correlated the increased GFP fluorescence to an increased expression of GnRH-mRNA, alluding to neurogenesis, this could have also been in conjunction with increased neural size. BPA was shown to decrease GnRH neural size in both directly exposed larvae (Inagaki et al., 2016a) and across generations (Inagaki et al., 2016b). Here we show that BPF at 0.25 μM decreased neural size of both TN and POA-GnRH neurons at 2 dpf, with no differences at 3 dpf. The higher doses of BPF altered only the sizes of TN-GnRH neurons at 3 dpf, indicating that BPF effects on neuron size are not only dose dependent but also have varying effects at the different GnRH neural populations over development. The POA-GnRH3 neurons directly affect pituitary release and gonadal hormone regulation. The TN-GnRH3 neurons have projections into different sensory systems and into the spinal cord, integrating information from across the brain (Oka, 2009). Changes in GnRH neuron activity/release, as an effect of altered GnRH neural size, could set the tone for the development of the reproductive neuroendocrine system, potentially affect sexual maturation, reproduction and even alter development of the following generation.

4.3. BPF has varying effects on the TN and POA-GnRH neural populations

We saw a difference in BPF effects on the TN and POA populations. What could constitute the differential response? GnRH neurons are located in three primary locations in the brain of most vertebrates and their ontogeny, physiology and function are different. In most vertebrates each of these populations express a different form of the GnRH gene, with *gnrh1* in the POA, *gnrh2* in the midbrain and, *gnrh3* in the TN associated with the olfactory bulb (Millar, 2005; Zohar et al., 2010). In zebrafish both the TN- and POA populations express *gnrh3* (Ramakrishnan et al., 2010; Zohar et al., 2010). While there is still debate in the field, there is evidence suggesting that the TN-GnRH neurons originate from the developing neural crest, while the POA-GnRH neurons derive from the pituitary placode (Whitlock, 2005; Zhao et al., 2013). Other studies suggest that the TN and POA both develop from the olfactory placode (Abraham et al., 2009). Evidence from teleost fish show that these populations are distinct both anatomically and physiologically (Oka, 2010). Previous work in the current transgenic zebrafish model system also showed that during development the TN- and POA populations have different rates of neuron growth, physiological properties and expression of synaptic vesicle markers (Zhao et al., 2013). Thus both the TN- and POA neurons at 2 and 3 dpf are undergoing rapid changes in both physiology and anatomical connections (Ramakrishnan et al., 2010; Zhao et al., 2013), and likely respond to endocrine disruption differently.

Here we show that BPF effects on TN- and POA- neurons vary with dose and age. While both the TN- and POA- GnRH3 neurons showed decreased neural size at 0.25 μM BPF exposure, this did not persist into 3 dpf. At the higher BPF concentrations the TN-GnRH3 population alone showed neural size differences at 3 dpf, with no differences at 2 dpf. Previous studies with BPA and BPS have shown that they exert varied effects at the TN- and POA populations at similar doses (Qiu et al., 2016). 100 ng/mL BPS increasing neuron numbers in the POA but not the TN at 25 hpf, while BPA at the same dose increased neural numbers in both POA and TN (Qiu et al., 2016). We did not observe any changes in neuron numbers at 2 or 3 dpf under all three BPF doses. This could be attributed to the age at which these populations were examined (close to 1dpf in the study by Qiu et al. (2016), as opposed to 2 and 3 dpf here). It could be that bisphenol effects on neurogenesis have maximal effects earlier in development. Also we were unable to accurately quantify the numbers of POA neurons at 2 and 3 dpf as they were numerous and at different depths of field (Fig. 1).

In medaka BPA has been shown to first increase GnRH3-GFP fluorescence at 3 dpf, which was then decreased at 5 dpf (Inagaki et al., 2016a). Several studies have reported that developing brains are sensitive to the exposure time of endocrine disruptors (Ramos et al., 2003; Vosges et al., 2010; Peretz et al., 2014). Kinch et al. (2015) reported that BPA increased neurogenesis in the zebrafish hypothalamus at 24 hpf but decreased it at 36 hpf. It has also been shown that bisphenols alter the brain aromatase expression via estrogen and androgen receptors in a time-sensitive manner (Chung et al., 2011; Kinch et al., 2015). Bisphenol effects on brain steroidogenesis, developmental changes occurring in this dynamic period of embryo development (Ramakrishnan et al., 2010; Zhao et al., 2013) and the fact that GnRH neurons have been shown to have an autocrine/paracrine feedback regulation (Karigo and Oka, 2013) could account for the differential BPF effects on the GnRH systems at 2 and 3 dpf.

4.4. Estradiol doses mimic the effects of BPF on GnRH neurons

This study demonstrates that estradiol and BPF treated embryos exhibited similar patterns of response. Aside from a 5 nM dose of estradiol, shown previously to elicit similar effects in other bisphenol studies (Ramakrishnan and Wayne, 2008; Inagaki et al., 2016a), two other concentrations 5 fold and 25 fold lower were used here. Another study in zebrafish larvae showed that 1 nM ethinyl estradiol stimulated estrogen receptor expression and aromatase expression similar to 1 μM BPF (Cano-Nicolau et al., 2016). Here we show that the effects of 0.2 nM estradiol exposure was similar to 0.25 μM BPF exposure, with decreases in TN- and POA- GnRH3 neuron size at 2dpf. The higher doses of estradiol mimicked the effects of higher BPF doses in both populations. These results suggest that BPF could act through an estrogenic pathway.

All subtypes of estrogen receptor (ER) mRNA have been reported in the POA and TN of the teleost brain (Zempo et al., 2013). BPF bound *in vitro* to ERα receptors with a lower affinity when compared to BPA. The binding affinity was reduced with ERβ1, and ERβ2 estrogen receptors. Although the direct mechanism is unknown, BPF effects were mitigated after co-application of BPF and ICI, an estrogen antagonist (Cano-Nicolau et al., 2016). Our results also showed that GnRH3 neurons of embryos co-exposed to 1 μM ICI with either 0.25 μM or 0.5 μM BPF showed no significant changes in neural size when compared to controls. Thus ICI, an estrogen antagonist, effectively mitigated BPF effects on the developing GnRH neural system.

4.5. Conclusions

This is one of the first papers to examine how low-dose, chronic exposure to BPF affects GnRH neurons in the developing embryo. Our data showed that BPF acted through an estrogenic pathway and caused

a decrease in GnRH neural size that was dose dependent. Given the similar effects of BPF to BPA, it is highly likely that the changes observed in the embryo persist through development, potentially affecting adult reproduction, behavior, with potential cross-generational effects.

Transparency document

The Transparency document associated with this article can be found in the online version.

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