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Sex specific impact of perinatal bisphenol A (BPA) exposure over a range of orally administered doses on rat hypothalamic sexual differentiation

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Abstract

Bisphenol A (BPA) is a high volume production chemical used in polycarbonate plastics, epoxy resins, thermal paper receipts, and other household products. The neural effects of early life BPA exposure, particularly to low doses administered orally, remain unclear. Thus, to better characterize the dose range over which BPA alters sex specific neuroanatomy, we examined the impact of perinatal BPA exposure on two sexually dimorphic regions in the anterior hypothalamus, the sexually dimorphic nucleus of the preoptic area (SDN-POA) and the anterioventral periventricular (AVPV) nucleus. Both are sexually differentiated by estradiol and play a role in sex specific reproductive physiology and behavior. Long Evans rats were prenatally exposed to 10, 100, 1000, 10,000 mg/kg bw/day BPA through daily, noninvasive oral administration of dosed-cookies to the dams. Offspring were reared to adulthood. Their brains were collected and immunolabeled for tyrosine hydroxylase (TH) in the AVPV and calbindin (CALB) in the SDN-POA. We observed decreased TH-ir cell numbers in the female AVPV across all exposure groups, an effect indicative of masculinization. In males, AVPV TH-ir cell numbers were significantly reduced in only the BPA 10 and BPA 10,000 groups. SDN-POA endpoints were unaltered in females but in males SDN-POA volume was significantly lower in all BPA exposure groups. CALB-ir was significantly lower in all but the BPA 1000 group. These effects are consistent with demasculinization. Collectively these data demonstrate that early life oral exposure to BPA at levels well below the current No Observed Adverse Effect Level (NOAEL) of 50 mg/kg/day can alter sex specific hypothalamic morphology in the rat.

Keywords

Endocrine disruption; Estrogen; Hypothalamus; Sexually dimorphic; Dopamine

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Conflict of interest

None.

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1. Introduction

Numerous attempts have been made to characterize the impact of early life bisphenol A (BPA) exposure on sexually dimorphic brain development in rodents because of growing concern that similar effects may occur in humans (He et al., 2012; Palanza et al., 2008; Richter et al., 2007; Wolstenholme et al., 2011). Two hypothalamic regions which have garnered considerable attention, because of their well characterized estrogen-dependent structural and functional sex differences (Simerly, 2002), are the sexually dimorphic nucleus of the preoptic area (SDN-POA) and the anteroventral periventricular (AVPV) nucleus. Males and females are born with the same number of neurons in both regions, but estradiol-mediated selective cell death mediated by ERα (Patchev et al., 2004) during neonatal life rapidly induces morphological sex differences (Wright et al., 2010). Remarkably, estradiol has opposite effects on cell survival in each region such that the SDN-POA is larger in males, and the AVPV is larger in females. The female AVPV also contains more dopaminergic neurons than males (Davis et al., 1996b; Simerly, 2002; Simerly et al., 1985a). Previous studies examining BPA-related impacts on SDN-POA and AVPV volume and composition have yielded discordant results (He et al., 2012; Kwon et al., 2000; Nagao et al., 1999; Patisaul et al., 2006, 2007; Rubin et al., 2006). Inconsistencies in the data likely result, at least in part, from experimental design differences including exposure duration, dose, route of BPA administration, and critical differences in neural structure between rats and mice (Bonthuis et al., 2010). To improve data continuity, study design-related guidelines for BPA research have recently been issued including statistical control for litter effects, examination over a wide dose range, use of oral administration, employment of concurrent positive controls, and performing all evaluations blinded to the exposure groups (Goodman et al., 2006; Hengstler et al., 2011; Hunt et al., 2009; Richter et al., 2007). Here we evaluated the impact of perinatal BPA exposure on SDN-POA and AVPV structure in rats of both sexes using these design guidelines to enhance the currently available data and provide results across a wider dose range than has been done previously.

In mammals, including humans, there are numerous structural and functional sex differences throughout the brain, particularly within the hypothalamus and surrounding structures (Bonthuis et al., 2010; De Vries, 2004; Simerly, 2002), which underpin physiological and behavioral sexual dimorphisms. The SDN-POA and AVPV are ideal regions to examine because the estrogendependent mechanisms by which they sexually differentiate, both structurally and functionally, are well understood and can be predictably manipulated by exogenous hormone administration (Davis et al., 1996a; Gilmore et al., 2012; Gorski et al., 1978, 1980; Sickel and McCarthy, 2000; Simerly, 1989; Simerly et al., 1985b; Yang et al., 2004). Thus, they are well defined targets for endocrine disrupting chemicals (EDCs) such as BPA. The SDN-POA is physically larger in males while the AVPV is larger in females; morphometric differences resulting from the presence or absence of perinatal estrogens (Bleier et al., 1982; Bloch and Gorski, 1988; Murakami and Arai, 1989). The specific cellular mechanisms by which estradiol can be pro-apoptotic in the AVPV but antiapoptotic in the SDN-POA remain to be fully characterized, but likely involve region specific proinflammatory cytokine and caspase signaling pathways (Wright et al., 2010).

The number of preoptic dopaminergic neurons, identified by the presence of tyrosine hydroxylase (TH; the rate-limiting enzyme for dopamine biosynthesis), is also sexually dimorphic in rats (Patisaul et al., 2006; Simerly et al., 1985b). In females, the region comprising the AVPV and the periventricular region just caudal to it contains approximately three times as many TH immunoreactive cells as the comparable male region (Simerly, 1989). Perinatal exposure to estradiol or an aromatizable androgen such as testosterone propionate results in masculinization of this region in females, and reduced numbers of immunoreactive TH (TH-ir) cells (Simerly, 1989; Simerly et al., 1985b). In contrast, the rat

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SDN-POA is 2–4 times larger in males than females (Gorski et al., 1978, 1980); a trait that develops in the first two weeks of life and results from a higher apoptotic rate in females (Davis et al., 1996a; Yang et al., 2004). In males, neonatal castration lowers circulating testosterone, from which neural estrogen is derived, and SDN-POA volume is consequently reduced (Davis et al., 1996a; Gorski et al., 1978, 1980; Sickel and McCarthy, 2000). Similarly, perinatal exposure to estradiol masculinizes the female SDN-POA resulting in increased volume (Dohler et al., 1982, 1984; Gorski et al., 1978). Calbindin-D28 (CALB), a calcium-binding protein and potential neuroprotectant, is a reliable marker to define the borders of the SDN-POA (Patisaul et al., 2007; Sickel and McCarthy, 2000). Thus, CALB immunolabeling (CALB-ir) was used here to identify both the borders of the SDN-POA and cell density within it.

These physical sex differences are reflective of functional ones. Both the SDN and AVPV are thought to play a role in the display of male sex behaviors (Rhees et al., 1999; Roselli et al., 2004), and lesion studies have demonstrated the critical importance of the AVPV in generating the preovulatory gonadatropin surge in females (Gerall et al., 1980; Wiegand and Terasawa, 1982). Although still highly controversial, in humans and sheep, a smaller SDN volume has been linked to the defeminization of sexual behavior and mate choice in males (Roselli et al., 2004). In rodents, early life BPA exposure has been shown to impact both male reproductive behavior (Jones et al., 2010) and female fertility (Cabaton et al., 2011); effects consistent with AVPV and SDN perturbation.

For the present study, Long Evans pups were perinatally exposed to a wide range of BPA doses (10, 100, 1000, 10,000 μg/kg/ day) through daily, non-invasive oral administration of dosedcookies to dams. This dose range spanned the current no observable adverse effect level (NOAEL) of 50 mg/kg bw/day and the current reference dose (tolerable daily intake) of 50 μg/kg bw/ day (Chapin et al., 2008; Geens et al., 2012; NTP, 1982). Despite well recognized metabolic differences between rats and humans, the lowest dose used here likely produces dam serum levels at the top end of, or just above, the human-relevant range (Doerge et al., 2011), although the boundaries of this range remain a point of contention (FAO/WHO, 2011; Hengstler et al., 2011; Sathyanarayana et al., 2011; Taylor et al., 2010; Teeguarden et al., 2011; Twaddle et al., 2010). Exposure to the pups (through gestation and lactation) is likely lower. Prior estimates (reviewed in (Chapin et al., 2008; FAO/WHO, 2011) suggest that gestational exposure is 1.6–18.5 times lower than that of the dam, and lactational transfer is even lower. Daily intake of BPA by humans is not well characterized but estimated to be approximately 0.4–1.4 μg/kg/ day (Chapin et al., 2008; FAO/WHO, 2011).

2. Methods

2.1. Animal care and exposure

Animal care and exposure of pregnant Long Evans (LEs) dams was conducted at the University of Rochester. All experimental procedures were performed in accordance with Society for Neuroscience guidelines and University of Rochester animal care and utilization committees (UCAR).

Sixty-four timed-pregnant LE female rats (Charles River, Raleigh, NC) were orally exposed to one of four BPA dose levels, BPA 10 ($n = 12$), BPA 100 ($n = 12$), BPA 1000 ($n = 10$), and BPA 10,000 ($n = 11$) μg/kg bw/day, corn-oil vehicle ($n = 11$), or 17β-estradiol ($n = 2$). This small number of estradiol-exposed dams was included to verify the sensitivity of LE rats to estrogenic compounds using this specific exposure paradigm. Prior studies have clearly established that exposure to 2 μg/kg bw/day 17β -estradiol during early development is sufficient to masculinize the size and CALB-ir content of the female rat

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SDN-POA (Dohler et al., 1984; Gilmore et al., 2012; Gorski et al., 1978) as well asTH-ircell number in the female AVPV (Patisaul et al., 2006; Simerly, 1989; Simerly et al., 1985a).

Dams arrived on gestational day (GD) 4 and were housed under a 12-h light cycle at 74 °F and 30–70% humidity in thoroughly washed polysulfone cages on woodchip bedding, fed Purina 5001 rodent chow (Purina Lab Diet, Richmond, IN), and provided with filtered tap water in glass water bottles ad libitum. BPA doses and the corn-oil vehicle were delivered daily to pregnant dams via a quartered, Nilla® Wafer cookie from GD 12 to postnatal day (PND) 10 using procedures similar to those described previously (Patisaul et al., 2013). Thus, developing rat pups were exposed *in utero* and during lactation for a total exposure period of 21 days.

Corn-oil vehicle or corn-oil/BPA dose (~0.2 cm^3 adjusted for bw) was applied daily to quartered, standard-sized (roughly 1″ to 1–1/4″ in circumference prior to quartering) Nilla® Wafers using a fresh, sterile 1cc syringe for each dose. The corn-oil/corn-oil BPA solution was readily absorbed by the wafer ensuring that the animal received the entire dose. Each animal had a separate, labeled weigh-boat in which the dosed cookie was transferred. The cookies were placed in the cage, away from the nesting location of the female by lifting the wire rack at a small angle (enough to accommodate the cookie) and dropped onto the bedding. Each dam was observed daily during this exposure regimen to ensure complete wafer consumption. The average time for dams to fully consume the wafer was approximately three minutes. All pups were weaned on PND 21 and randomly assigned to one of four experimental groups. Four males and four females per litter were used to generate the data.

2.2. Tissue collection and preparation

Animals were sacrificed between PNDs 65–68. Animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.9% NaCl followed by 400 ml 4% paraformaldehyde in 0.01 M sodium phosphate buffer (pH 7.4). Females were sacrificed in estrous (verified by vaginal cytology (Becker et al., 2005)) and weight was recorded for all animals at the time of sacrifice. Brains were removed and postfixed in 30% sucrose/4% paraformaldehyde for 3–4 h, then cryoprotected in 30% sucrose/ PBS solution for 24–72 h (Hoffman and Le, 2004). Brains were rapidly frozen on dry ice, shipped to NCSU for processing and stored at −80 °C. Each brain was coronally sectioned at 50 μm using a freezing slide microtome, divided into four series of alternating sections comprising the SDN-POA and AVPV and stored free-floating in a cryoprotectant antifreeze solution (30% sucrose, 30% ethylene glycol, 10% polyvinylpyrrolidone, 5% glycerol in 0.1 M sodium phosphate buffer) at −20 °C until staining (Hoffman and Le, 2004).

2.3. Immunohistochemistry

SDN-POA sections were immunolabeled for CALB, and sections comprising the AVPV and the periventricular region just caudal to it were immunolabeled for TH as detailed in our prior publications (Patisaul et al., 2006, 2007). Selected sections were 100 μm apart in the SDN-POA, and 200 μm in the AVPV region. Briefly, all sections were thoroughly washed at 4 °C in 0.02 M potassium phosphate buffered saline (KPBS), and endogenous peroxidase activity was quenched with a 15min wash in 0.5% H_2O_2 in DK-LKPBS (2% normal donkey serum and 0.3% Triton X in 0.2 M KPBS). The sections were then washed and incubated in DK-LKPBS overnight at 4 °C. SDN-POA sections were incubated in a primary antibody solution directed against CALB (Mouse Monoclonal Anti-Calbindin-D-28K (Cat # C9848, Sigma, St. Louis, MO), 1:100,000 in DK-LKPBS; Sigma, St. Louis, MO), and AVPV sections were incubated in a primary antibody directed against TH (Mouse Monoclonal Anti-Tyrosine Hydroxylase (Cat # 22941, Immunostar, Hudson, WI), 1:80,000 in DK-

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LKPBS) for 72 h at 4 °C. All sections were then washed and placed in a biotinylated donkey anti-mouse immunoglobulin G (IgG) secondary antibody (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) for 90 min at room temperature. The signal was amplified using an avidin-biotin complex kit (Vector Labs, Burlingame, CA) and developed using DAB chromagen (Vector Labs, Burlingame, CA). After a final set of washes, the sections were serially mounted onto Fisherbrand Superfrost Plus slides (Fisher, Pittsburgh, PA) and allowed to dry overnight. The sections were then dehydrated via washes of increasing ethanol stringency and cleared in xylene (Fisher, Pittsburgh, PA) for two hours. Slides were coverslipped with DPX mountant (Electron Microscopy Services, Hatfield, PA).

2.4. Quantification of CALB-ir and SDN-POA volume

Quantification of SDN-POA CALB-ir cells and determination of SDN-POA nuclear volume, was accomplished via unbiased stereology (Glaser and Glaser, 2000; Schmitz and Hof, 2005) as we have done previously (Patisaul et al., 2007). All analyses were done using Stereologer™ (Stereology Resource Center, Inc., MD) on a Leica DM2500P scope (Leica Microsystems, Wetzlar, Germany). The borders of the SDN-POA were clearly defined by CALB immunolabeling and confirmed using the Adult Rat Stereotaxic Atlas (Paxinos and Watson, 2007). For each section, SDN-POA borders were traced at low magnification (5×) and then analyzed at high magnification (63×). Volume was calculated using Calviari's Principle. CALB-ir cells were counted using the optical fractionator. The complete nucleus was contained within 2–3 sections per animal. The final post-processing tissue thickness of the sections was measured to be approximately 22.7 μm, therefore the frame height was set below that threshold at 20 μm with a guard height of 1 μm. To quantify the dense population of cells, the frame area was set at 15 μm^2 (3.873 μm × 3.873 μm), and framing spacing was 50 μm. The volume of the CALB-ir subregion of the SDN was quantified with a region point counting area per point of 1000 μm^2. The mean coefficient of error for CALB-ir cells counted with the optical fractionator was 0.14 and for CALB-SDN volume was 0.09. Images were captured using a Qimaging Retiga 2000R 12-bit color camera (QImaging, Surry, British Columbia, Canada) mounted on a Leica DM5000B scope (Leica Microsystems, Wetzlar, Germany) using MCID Core Image software program (InterFocus Imaging Ltd., Cambridge, England).

2.5. Quantification of tyrosine hydroxylase (TH) immunoreactivity in the AVPV

Quantification of TH-ir was done with the optical fractionator as described above for CALB-ir cells in the SDN-POA. TH-ir cells were contained within 3–4 sections per animal. The final postprocessing tissue thickness of the sections was measured to be approximately 22.7 μm, therefore the frame height was set at 20μm with a guard height of 1 μm. The frame area was 2500 μm^2 (50 μm × 50 μm), and framing spacing was 50 μm. The region point counting area per point was set at 1000 μm^2. The mean coefficient of error for TH-ir nuclei counted with the optical fractionator was 0.09. Images were captured using a Qimaging Retiga 2000R 12-bit color camera (QImaging, Surry, British Columbia, Canada) mounted on a Leica DM5000B scope (Leica Microsystems, Wetzlar, Germany) using MCID Core Image software program (InterFocus Imaging Ltd., Cambridge, England).

2.6. Statistical analysis

Data analysis was performed using published guidelines established for assessing low-dose endocrine disruptor data (Haseman et al., 2001). Control and 17β-estradiol exposed females were compared by a Student's *t*-test (pooled variance) for each measure to confirm the sensitivity of the animal model to the masculinizing influence of estradiol. For each endpoint, control and BPA exposed animals of both sexes were compared by two-way analysis of variance (ANOVA) with exposure group and sex as factors, and followed up with a one-way ANOVA within sex. Significant effects were followed up by protected

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Fisher's least significant difference (LSD) post hoc analysis. Two sample *t*-tests (separate variance) were performed within each exposure group to address if sex difference was preserved between male and female groups. All analyses were completed using SYSTAT software (SYSTAT, Systat Software Inc., Richmond, CA) and in all cases effects were considered significant at p 0.05.

3. Results

3.1. Confirmation of strain sensitivity

Both regions were completely masculinized by 17β-estradiol, observations which confirm the sensitivity of the LE rat to oral estrogen during this critical window of development and demonstrate that it is an appropriately sensitive animal model for examining BPA effects. Estradiol exposed females (n = 2) had significantly fewer AVPV TH-ir neurons then unexposed controls ($t(9.000) = 3.820$, p 0.004). Similarly, SDN-POA volume ($t(7.000) = 7.763$, p 0.001) and CALB-ir cell number ($t(7.000) = 7.763$, p 0.02) was significantly increased in estradiol exposed females.

3.2. Impact of BPA on SDN-POA volume and CALB-ir

As anticipated (Davis et al., 1996a; Dohler et al., 1984; Gorski et al., 1978; Patisaul et al., 2007; Sickel and McCarthy, 2000), control males had both a significantly larger SND-POA ($t(9.310) = 6.251$, p 0.001) and a greater number of CALB-ir cells within it ($t(9.812) = 4.201$, p 0.002) compared to control females. Two-way ANOVA revealed an interaction between exposure group and sex for both SDN volume ($F(4,84) = 5.719$, p 0.001) and CALB-ir cell numbers ($F(4,81) = 3.890$, p 0.006). Significant effects of exposure were only present in males. There was a main effect of BPA exposure on male SDN-POA volume ($F(4,38) = 6.557$, p 0.001; Fig. 1), and post hoc analysis revealed that SDN-POA volume was significantly smaller in all BPA exposed groups compared to unexposed controls (Fig. 3). Volumetric effects were greatest in the BPA 10 μg/kg/day (p 0.001) and BPA 100 μg/kg/day (p 0.001) groups, with mean volume decreased 45% and 39%, respectively. There was also a main effect of exposure on the number of CALB-ir cells in the male SDN-POA ($F(4,40) = 3.9445$, p 0.009) with numbers significantly decreased in all BPA exposed groups compared to the unexposed controls (p 0.003 for all) except the BPA 1000 group ($p = 0.07$; Fig. 2). Two sample t-tests within each group revealed that the sex difference in SDN-POA volume was preserved in all exposure groups (BPA 10: $t(13.577) = 3.174$, p 0.007; BPA 100: $t(17.424) = 3.631$, p 0.002; BPA 1000: $t(10.013) = 7.415$, p 0.001; BPA 10,000: $t(8.399) = 4.098$, p 0.003). The sex difference in CALB-ir cell density, however, was only preserved in the BPA 1000 exposure group ($t(11.552) = 2.376$, p 0.036), and lost in the other groups.

3.3. Impact of BPA on AVPV TH-ir

As expected (Davis et al., 1996b; Patisaul et al., 2006; Simerly et al., 1985a), AVPV TH-ir was sexually dimorphic in the unexposed controls ($t(15.201) = 4.807$, p 0.001), with females having nearly twice as many TH-ir neurons than males (Fig. 4). Two-way ANOVA revealed a main effect of sex ($F(4,92) = 4.694$, p 0.002) and a main effect of exposure ($F(1,92) = 42.214$, p 0.001) but no significant interaction between sex and exposure. Because TH-ir is sexually dimorphic, subsequent analyses were performed within sex. One way ANOVA within sex revealed a main effect of BPA exposure on AVPV TH-ir cell numbers in females ($F(4,52) = 3.142$, p 0.02) and males ($F(4,39) = 4.153$, p 0.007) (Fig. 5). TH-ir cell numbers were significantly lower in all BPA exposed females (p 0.05 for all) with the exception of the BPA 1000 group ($^{\dagger}p$ 0.06). In males, TH-ir cell numbers were only significantly impacted in the BPA 10 (p 0.03) and BPA 10,000 (p 0.013) exposure groups, with fewer TH-ir cells compared to controls. Two sample t-tests within

each exposure group revealed that the sex difference in TH-ir cell density was preserved in all exposure groups (BPA 10: t(19.743) 2.779, p 0.01; BPA 100: t(16.069) = 2.423, p 0.03; BPA 1000: t(16.270) = 2.670, p 0.02; BPA 10,000: t(18) = 2.943, p 0.01).

4. Discussion

Perinatal BPA exposure via oral exposure to the dam altered the structure and composition of the rat SDN-POA and AVPV within a dose range encompassing the current reference dose of 50 μg/ kg bw/day. Effects were region and sex specific with evidence of demasculinization in the male SDN-POA, and evidence of defeminization in the female AVPV. All effects were significant at the lowest dose employed (10 μg/kg bw/day). Despite well recognized metabolic differences between rats and humans, this dose likely produces serum levels at the top end of the humansrelevant range (Chapin et al., 2008; Doerge et al., 2011), although the bounds of this range remain unresolved (FAO/WHO, 2011; Hengstler et al., 2011; Sathyanarayana et al., 2011; Taylor et al., 2010; Teeguarden et al., 2011; Twaddle et al., 2010). Importantly, the effects occurred at exposure levels below those needed to increase uterine weight, suggesting that the brain may be a more sensitive endpoint when considering the potential of BPA, and other endocrine disruptors, of interfering with the organizational role of estrogen on hormone sensitive structures. Why the brain is more responsive than the uterus is unclear but differences in the milieu of co-factors and co-repressors needed for estrogen dependent transcription, activation of non-classical signaling pathways, or induction of epigenetic changes which enhance estrogen receptor activity are plausible (McCarthy et al., 2009; Yeo et al., 2013). Collectively, these data suggest that steroid hormone sensitive brain regions may be vulnerable to endocrine disruption by BPA resulting in altered sex specific morphology.

SDN-POA effects were only observed in males, which is consistent with a prior report from our research group (Patisaul et al., 2007). An important difference, however, is the direction of the effect. In the prior study, male Sprague Dawley (SD) rats were subcutaneously injected with 250 μg BPA every 12 h over the first two days of life, which is approximately equivalent to 42 mg/kg bw per day, and 4-fold higher than the highest dose used in the present study. At this higher dose, SDN-POA volume was unchanged but the number of CALB-ir cells was significantly increased. Other research groups have also observed that SDN-POA volume is unaltered by high dose developmental BPA exposure. For example, neonatal injections of 300,000 μg/kg bw/day (Nagao et al., 1999), or perinatal oral exposure to 200,000–400,000 μg/kg bw/day failed to alter male SDN-POA volume in SD rats (Kwon et al., 2000; Takagi et al., 2004). Here, however, we found that low dose perinatal BPA exposure, reduced, rather than increased, CALB-ir neuron numbers, and male SDN-POA volume was also diminished. These effects were most pronounced at the lowest doses used (10 and 100 μg/kg/day bw orally).

One possible explanation for these dose-dependent differences on SDN-POA morphometrics across studies is that the direction of the effect reverses across the dose curve; with demasculinization occurring at low doses and resistance to change or subtle hypermasculinization occurring at higher doses. Collectively, our data suggest that the inflection point is around 100 μg/kg bw/day. The mechanism by which this dose-specific response reverses direction remains unclear but likely involves a dose-dependent interaction with estrogen signaling. In the female SDN-POA, the masculinizing effect of estradiol can be induced by selective agonism of ERa but not ERb, demonstrating the importance of ERa for enhancing volume (Patchev et al., 2004). BPA may inhibit estrogen signaling at low doses, resulting in increased apoptosis, but augment estrogen signaling at high doses, thereby enhancing cell preservation in this region.

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The dose-dependent volumetric change hypothesis posed above is supported by all available data published to date on the rat SDN-POA, with the exception of a recent study, which reported evidence for hypermasculinization at 2.5 μg/kg/day bw; a dose lower than any used in the present study (He et al., 2012). Key design elements in that study may account for this discrepant finding, one of which is the route of oral dosing employed. For their study, He and colleagues (2012) used orogastric gavage (to the dams during gestation and then directly to the pups until weaning) which is a popular oral exposure route because it ensures precise dosing, but can be stressful to the animals (Balcombe et al., 2004). Significant differences in pup weight and survival between litters born to vehicle gavaged and naïve (ungavaged) controls were observed (Ferguson et al., 2011), suggesting that gavage-related stress might have confounded toxicant-related effects on hormone sensitive brain endpoints, including the SDN-POA. Subsequent work will be needed to more clearly establish how early life stress, such as the stress associated with gavage or other forms of handling, interact with exposures to endocrine disruptors and other toxicants to alter brain morphology and sex specific organization.

For the present study, BPA was administered to the dams using a food treat, which eliminates the potential confound of dosing stress but relies on lactational transfer for the pups to be effectively dosed after birth. In humans, BPA has been shown accumulate in fetal liver tissue (Nahar et al., 2012), serum, and amniotic fluid (Engel et al., 2006; Ikezuki et al., 2002) demonstrating the capacity for gestational exposure. Of note, fetal BPA concentrations were shown to have a greater variance in retention time, greater mean retention time, and a longer terminal half-life than that of dams (e.g., half-life from 6 to 48 h collection was approximately 37.2 times greater in fetuses than in dam blood) (Chapin et al., 2008; FAO/WHO, 2011). In rats, however, lactational transfer of BPA appears to be less efficient, with pup serum concentrations 300 times lower than the exposed dams (Chapin et al., 2008; Doerge et al., 2010). It is therefore possible that, in the present study, BPA exposure occurred primarily during gestation. If so, then these data would suggest that the observed decreases in male SDN-POA volume and CALB content result primarily from exposure prior to birth. In female rats, postnatal exposure to diethylstilbestrol (DES) is more effective at masculinizing the SDN-POA than prenatal exposure (Tarttelin and Gorski, 1988). Subsequent work established that the hormone-sensitive period for SDN-POA sexual differentiation begins on GD 18 (Rhees et al., 1990a) and ends abruptly on PND 5 (Rhees et al., 1990b), with males being more sensitive to hormone manipulation than females. In the present study, lower BPA exposure during postnatal life due to poor lactational transfer could account for why no statistically significant effects of BPA exposure were observed in the female SDN-POA. Moreover, in the study reporting increased male SDN-POA volume at 2.5 μg/kg bw/day (He et al., 2012), animals were exposed during gestation but then also gavaged directly up to the point of sacrifice at weaning. This post-natal exposure may be why low dose BPA enhanced SDN-POA volume in their exposure paradigm but not ours.

In contrast to the SDN-POA, effects in the AVPV were observed in both sexes. In the male AVPV, hypermasculinization of TH-ir numbers occurred only in the lowest and highest BPA exposure groups (10 μg/kg/day and 10,000 μg/kg/day) suggesting a non-monotonic dose response. In females, evidence of masculinization was observed across all BPA exposed groups, but did not achieve statistical significance in the BPA 1000 group ($^{\dagger}p$ 0.06). These results are in accord with a prior study, using CD1 mice exposed via mini-pumps to 25 or 250 ng/kg bw/BPA from GD 16 to PND 16, which also found masculinization of the female AVPV (Rubin et al., 2006). Similar to what was observed in the SDN-POA, at higher doses, the sex specific outcome appears to reverse. We have previously shown that s.c. injection of 250 μg BPA every 12 h over the first two days of life, has no effect on total TH-ir numbers in females, but significantly increases TH-ir levels in males (Patisaul et al., 2006). This effect is consistent with the hypothesis that BPA blocks

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estrogen action at low doses but augments it at higher doses. Interactions with ERβ may also play a role. Neonatal exposure to an ERβ selective agonist masculinizes the female AVPV (Bodo et al., 2006; Patchev et al., 2004), and ERβKO males have been shown to possess an abnormally high number of TH-ir neurons in the AVPV (Bodo et al., 2006). At birth, ERβ expression is higher in the male AVPV, but this sex difference equalizes by PND 2 (Cao and Patisaul, 2011). Exposure to 50 μg/kg bw or 50mg/kg bw/BPA by s.c. injection from birth to PND 2 significantly eliminates AVPV ERβ expression levels in both sexes by PND 4 suggesting that altered ER expression could underlie the morphometric changes reported here.

Prior studies exploring the impact of early life BPA exposure on brain development and gene expression have produced inconsistent and conflicting data (Palanza et al., 2008; Richter et al., 2007; Wolstenholme et al., 2011) which has confounded risk assessment. Although a number of study design elements, including differences in exposure duration, dose, route of BPA administration, and critical species differences in neural structure between rats and mice (Bonthuis et al., 2010), likely account for the discordance in the literature, concerns about the sensitivity of some rat strains to gonadal steroid-derived effects have also been raised (Long et al., 2000; Richter et al., 2007; Steinmetz et al., 1998). Thus, we exposed a very small group of females ($n = 2$) to 17β-estradiol to expressly confirm the estrogen sensitivity of the LE rat strain used for this study. As expected based on numerous prior studies using LE rats (Cao et al., 2012; Fader et al., 1998; Ford et al., 2004; Laws et al., 2000), all endpoints were fully masculinized in the estradiolexposed females, including SDN-POA volume (Dohler et al., 1984; Gilmore et al., 2012; Gorski et al., 1978; Patchev et al., 2004) and decreased TH-ir cell number in the AVPV (Patisaul et al., 2006; Simerly, 1989). Assessment of strain sensitivity by including a concurrent positive control for the predicted effect is critical when attempting to evaluate the impact of BPA, especially when no significant effects are observed.

The potential for human-relevant exposure to result in adverse health outcomes remains a subject of active debate (Beronius et al., 2010; Hengstler et al., 2011; Vandenberg et al., 2009). It is important to emphasize that species differences make organizational neuroendocrine effects in animals difficult to apply to human risk assessment. In humans, androgen rather than estrogens is thought to be most important for masculinizing the brain during development (Grumbach, 2002; Wallen, 2005). This difference makes it challenging to infer how endocrine disrupting compounds, like BPA, might impact the sexual differentiation of the human hypothalamus or other brain regions. Our data reveal that the rat anterior hypothalamus is sensitive to endocrine disruption by BPA at oral doses below the current reference dose, suggesting that neural effects in humans are plausible. Animals were exposed perinatally, a critical period that is entirely prenatal in humans. Prenatal exposure to BPA has been demonstrated in humans (Braun et al., 2011,2009; Calafat et al., 2008) and associated with elevated anxiety and hyperactivity in young girls. Collectively, these epidemiological data support the possibility that developmental BPA exposure has adverse, sex specific, neural effects in humans.

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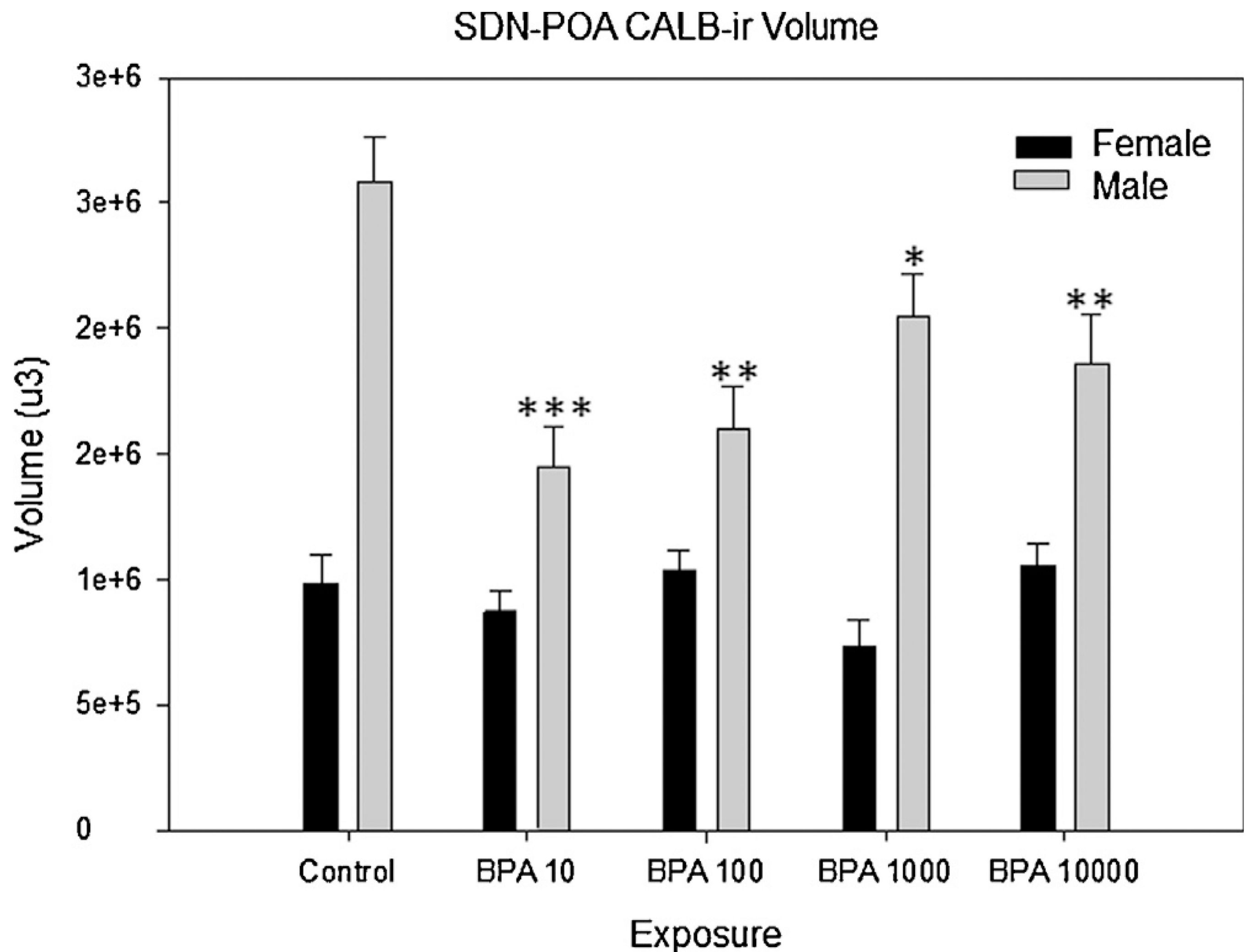

Fig 1.

Bisphenol-A exposure on SDN-POA CALB-ir volume in adult male and female rats. There was a main effect of BPA exposure on the volume of the SDN-POA observed between control male and all male exposure groups (p 0.001). No significant change was observed in female exposure groups. All exposure groups are in μg/kg/day. S.E. is indicated by error bars. Control M, F ($n = 8, 7$); BPA 10 M, F ($n = 10, 13$); BPA 100 M, F ($n = 9, 13$); BPA 1000 M, F ($n = 9, 7$); BPA 10,000 M, F ($n = 7, 11$); statistically significant change within exposure groups as compared to control animals within the same sex are as indicated: *p < 0.01–0.05, **$p < 0.001$– 0.01, ***$p < 0.001$.

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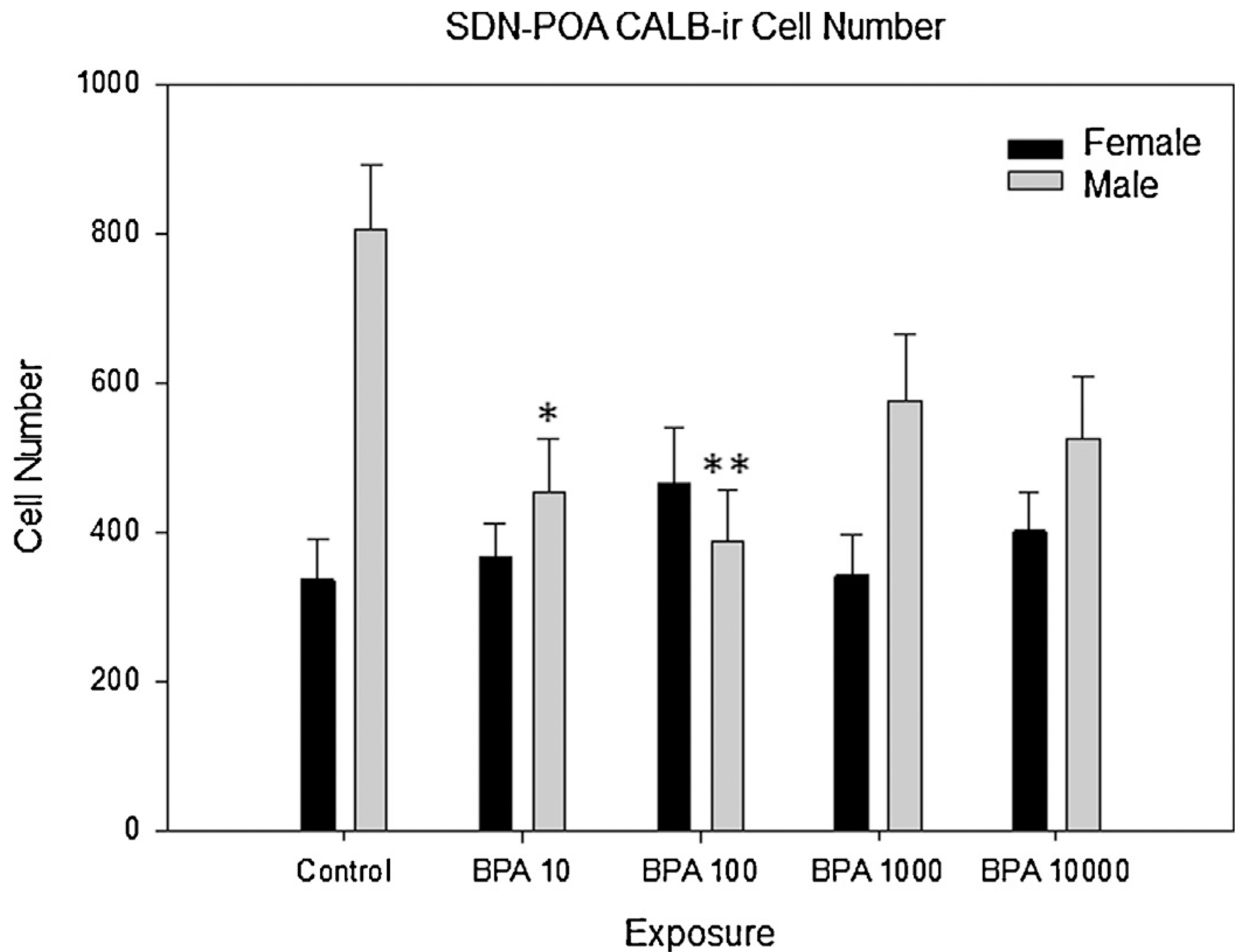

Fig 2.

Bisphenol-A exposure on SDN-POA CALB-ir cell number in adult male and female rats. There was a significant overall effect of BPA exposure (p 0.009) in male exposure groups BPA 10 and BPA 100 as compared to control males. All exposure groups are in μg/kg/day. S.E. is indicated by error bars. Control M, F ($n = 7, 9$); BPA 10 M, F($n = 11,13$); BPA 100 M, F ($n = 12, 5$); BPA 1000 M, F($n = 7, 9$); BPA 10,000 M, F ($n = 8, 10$); statistically significant change within exposure groups as compared to control animals within the same sex are as indicated: *$p < 0.01$–0.05, **$p < 0.001$–0.01, ***$p < 0.001$, †$p = 0.072$.

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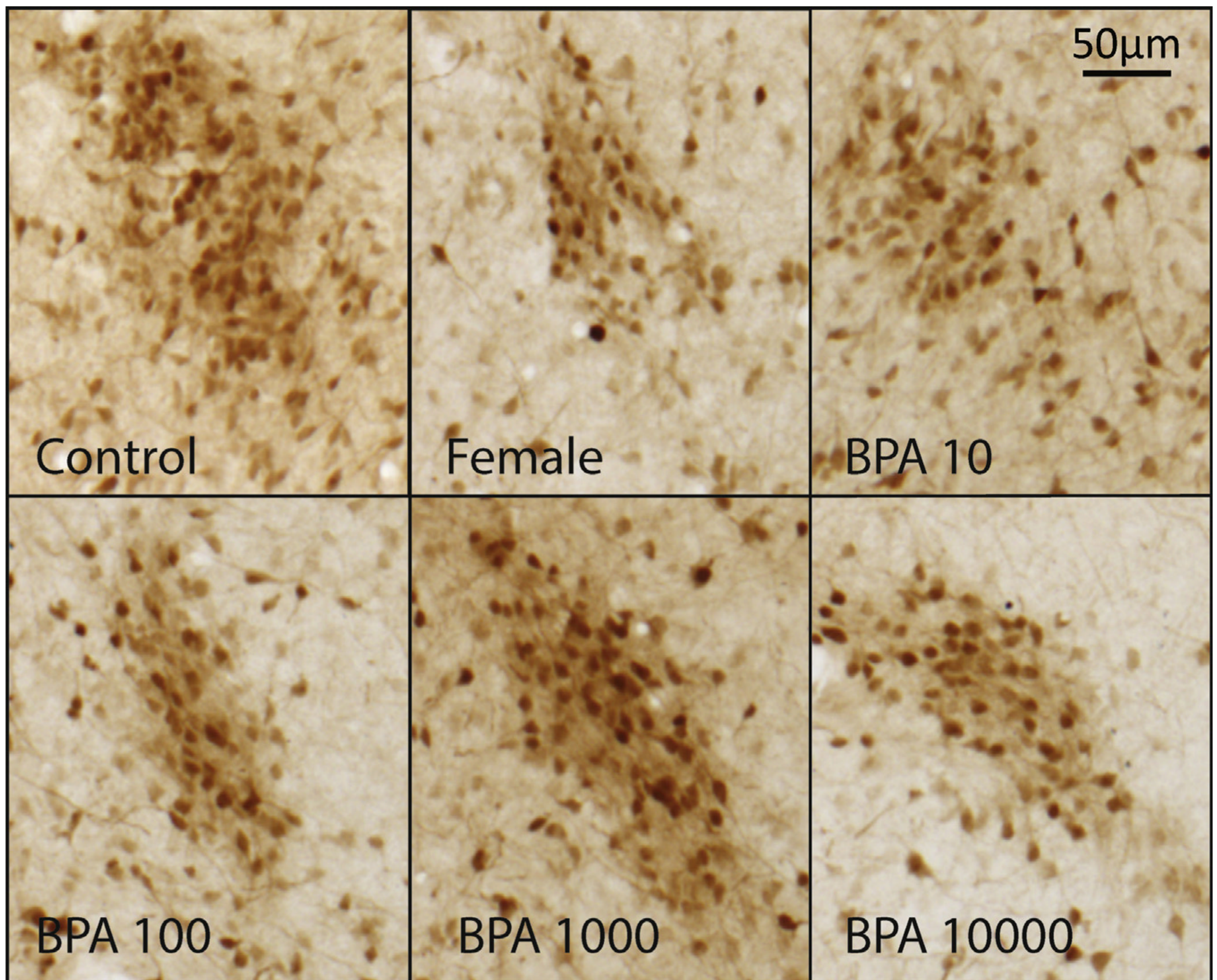

Fig 3.
SDN-POA CALB-ir cells in exposed males compared to control males and females. Representative images (10×) depicting the density and distribution of CALB-ir within the SDN-POA of vehicle treated males (control) and females (females) and males exposed to BPA (10, 100, 1000, and 10,000 μg/kg bw/day).

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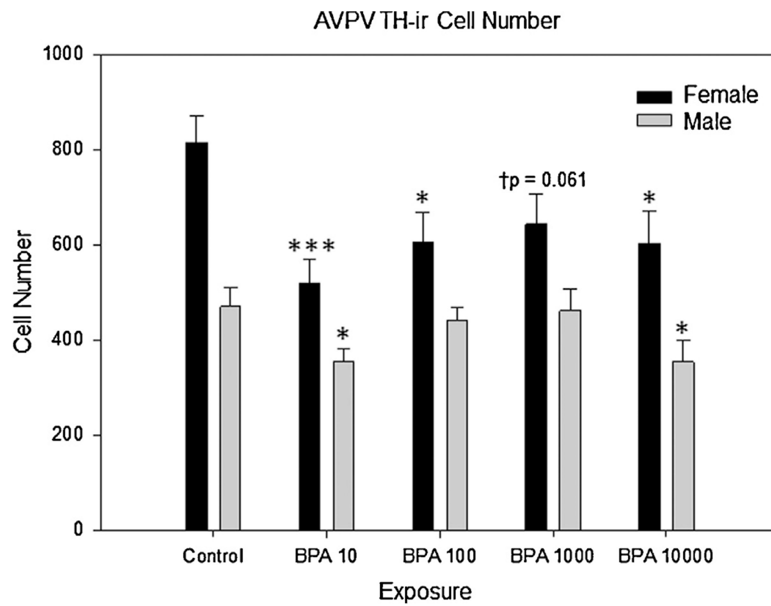

Fig 4.

Early developmental bisphenol-A exposure on AVPV-TH-ir cell number in adult male and female rats. There was a main effect of BPA exposure on AVPV-TH-ir cell number observed between control female and all female exposure groups (p 0.022), with the exception of BPA 1000 ($^{\dagger}p = 0.061$). In male exposure groups, BPA exposure significantly effected (p 0.007) AVPV-TH-ir cell number in BPA 10 and BPA 10,000 groups as compared to control males. All exposure groups are in μg/ kg/day. S.E. is indicated by error bars. Control M,F($n = 9,10$); BPA 10M,F($n = 9,13$); BPA 100 M, F ($n = 10, 12$); BPA 1000 M, F ($n = 8, 11$); BPA 10,000 M, F ($n = 8, 11$); statistically significant change within exposure groups as compared to control animals within the same sex are as indicated: *$p <$ 0.01–0.05, ***$p < 0.001$.

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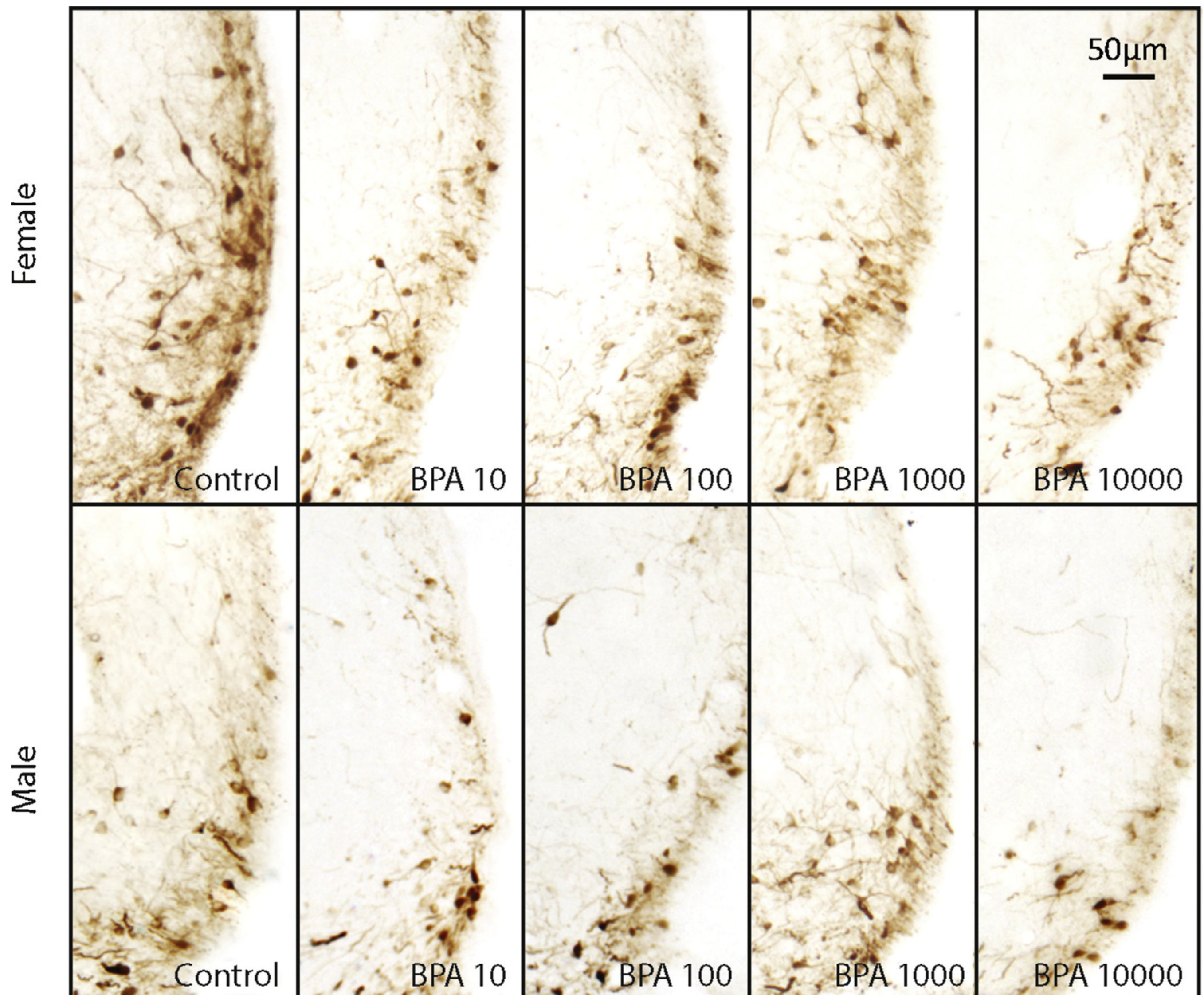

Fig 5.

AVPVTH-ir cells in males (top panels) and females (bottom panels). Representative images (10×) depicting the density and distribution ofTH-ir neurons in the vehicle controls and BPA exposed (10, 100, 1000, and 10,000 μg/kg bw/day) animals of both sexes.