

Neonatal Bisphenol-A Exposure Alters Rat Reproductive Development and Ovarian Morphology Without Impairing Activation of Gonadotropin Releasing Hormone Neurons

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Short Title: Hypothalamus not defeminized by Bisphenol-A

Summary Sentence: Neonatal exposure to Bisphenol-A induces early puberty, anestrus, and ovarian malformations but does not appear to defeminize the hypothalamus.

Keywords: hypothalamus, AVPV, endocrine disruptors, GnRH, corpora lutea, estrogen, PPT, development, disruption, ovary, gonad, estrogen receptor, development, lordosis, ER α , ESR1

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Abstract

Developmental exposure to endocrine disrupting compounds (EDCs) is hypothesized to adversely affect female reproductive physiology by interfering with the organization of the hypothalamic-pituitary-gonadal (HPG) axis. Here we compared the effects of neonatal exposure to two environmentally relevant doses of the plastics component bisphenol-A (BPA; 50 μ g/kg and 50 mg/kg), with the ESR1 (formerly known as ER α) selective agonist 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; 1 mg/kg) on the development of the female rat hypothalamus and ovary. An oil vehicle and estradiol benzoate (EB, 25 μ g) were used as negative and positive controls. Exposure to EB, PPT or the low dose of BPA advanced pubertal onset. 67% of females exposed to the high BPA dose were acyclic by 15 weeks after vaginal opening compared to 14% of those exposed to the low BPA dose, all of the EB and PPT treated females and none of the control animals. Ovaries from the EB treated females were undersized and showed no evidence of folliculogenesis, while ovaries from the PPT treated females were characterized by large antral-like follicles which did not appear to support ovulation. Severity of deficits within the BPA treated groups increased with dose and included large antral-like follicles, and lower numbers of corpora lutea. Sexual receptivity, examined after ovariectomy and hormone replacement, was normal in all groups except those neonatally exposed to EB. FOS induction in hypothalamic gonadotropic (GnRH) neurons following hormone priming was impaired in the EB and PPT treated groups, but neither of the BPA treated groups. Our data suggest that BPA disrupts ovarian development but not the ability of GnRH neurons to respond to steroid positive feedback.

Introduction

Bisphenol-A (BPA) initially entered commercial development in the 1930s as a synthetic estrogen [1], but is now used primarily in the production of polycarbonate plastic products, epoxy resins and the linings of soda and soup cans. The United States Centers for Disease Control recently estimated that nearly all Americans have detectable levels of BPA in their bodies and that children have higher levels than adults [2, 3]. Infants in neonatal intensive care units may have notably high exposure to BPA, presumably from its use in medical devices [4]. Newborns can also be exposed through lactational transfer [5]. Fetal exposure is also highly likely because relatively high levels in umbilical cord blood and fetal plasma indicates that BPA fails to bind to the estrogen sequestering protein alpha fetoprotein and can cross the placenta [6, 7]. In multiple species, exposure to BPA during the pre- or postnatal period has been shown to impact female reproductive physiology including the timing of pubertal onset and the induction of an early, persistent estrus [8-10]. Presumably, BPA produces its effects by interfering with one or both of the primary forms of the estrogen receptor (ESR1 or ESR2, formerly known as ER α and ER β) within the hypothalamic-pituitary-gonadal (HPG) axis but it is not well understood through which ER BPA primarily acts *in vivo*. In the present study, we took a comparative approach and examined the effects of neonatal exposure to BPA or an ESR1 selective agonist on the structure and function of the adult hypothalamus and ovary in female rats. We have previously shown that exposure to the ESR1 specific agonist 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) but not the ESR2 specific agonist diarylpropionitrile (DPN) during the neonatal period can defeminize the female rat hypothalamus and impair steroid positive feedback [11]. Here we sought to determine if neonatal exposure to BPA could similarly impact hypothalamic organization, a result which would then implicate a mechanistic role for ESR1 in endocrine disruption by BPA in the female hypothalamus.

Within the HPG axis, reproductive maturation and function is coordinated by the release of gonadotropin releasing hormone (GnRH) [12, 13]. The neural components of the HPG axis that regulate GnRH secretion are sexually differentiated by endogenous gonadal hormones, primarily estradiol, through a series of gestational, pre- and perinatal critical periods [14]. GnRH release is regulated through feedback effects of gonadal steroids. In both males and females, GnRH secretion is suppressed by steroid negative feedback, the signal for which is thought to arise from the arcuate nucleus of the hypothalamus [15, 16]. In females, however, GnRH release is augmented once per cycle by estrogens. This positive feedback potentiates the surge in GnRH and, subsequently, luteinizing hormone (LH) that precedes ovulation [17]. In rats, this process is now thought to be mediated within the anterior ventral periventricular nucleus (AVPV) of the hypothalamus [15, 16, 18].

In rodents, sexual differentiation of this feedback circuit takes place, primarily, during the neonatal period and has been shown to be particularly sensitive to disruption by hormones or hormone-like compounds [11, 19, 20]. It is well established that the administration of steroid hormones including testosterone or estradiol benzoate (EB), during the neonatal critical period can defeminize the female rodent brain, while castration can effectively prevent defeminization of the male rodent brain [21-23]. Thus, in males castrated as neonates, the potential for estrogen to evoke a GnRH surge is preserved while, conversely, in females neonatally exposed to estrogens, this capacity is diminished or lost. Exposure to sex steroids or endocrine disrupting compounds (EDCs) during the neonatal critical period can also advance puberty and alter ovarian morphology [15, 24-26]. The present experiments targeted this neonatal critical window, which corresponds to approximately the third trimester of gestation in humans [27]. In a prior study, we demonstrated that neonatal administration of the phytoestrogens genistein (GEN) and equol (EQ) can advance puberty and induce an early, persistent estrus [11]. This disruption of female reproductive physiology was accompanied by impaired GnRH activation (as measured by the colocalization of GnRH and FOS immunoreactivity), suggesting that perturbation of the hormone dependent organization of steroid positive feedback circuits within the hypothalamus was a potential mechanism for the effects on pubertal timing and ovarian cyclicity. Animals neonatally exposed to the ESR1 specific agonist PPT but not the ESR2 specific agonist DPN, also displayed an early persistent estrus and inhibited FOS induction

in GnRH neurons following hormone priming, demonstrating that ESR1 plays a critical role in the defeminization of the hypothalamus by estrogens. By extension, ESR1 might play a mechanistic role in the disruption of hypothalamic organization by EDCs such as BPA. Reduced colocalization of GnRH and FOS immunoreactivity following neonatal exposure to BPA would potentially indicate that BPA can interact with ESR1 in the hypothalamus during development, a finding that would provide key information about the possible mechanism(s) by which BPA affects the organization of the female reproductive system.

Developmental exposure to estrogenic EDCs can induce additional effects elsewhere in the HPG axis, such as the ovary [10, 28, 29]. Previous studies have shown that perinatal exposure to GEN can lead to altered ovarian differentiation characterized by multiocyte follicles and reduced fertility in mice [25]. In contrast, relatively little is known about how exposure to BPA, at doses considered relevant for human risk assessment, over the neonatal critical period alone, can affect ovarian development and function. Therefore, the present study had two main goals: (1) to determine if advanced puberty and impaired estrus function in female rats following neonatal exposure to one of two relatively low doses of BPA is associated with impaired sexual receptivity and FOS induction in hypothalamic-GnRH neurons and (2) to characterize ovarian morphology in the adult following neonatal BPA exposure.

Because ERs are expressed in all three HPG components, not just the hypothalamus, disruption by BPA could potentially occur anywhere within it, including the pituitary, the ovary, the brain, or any combination of the three. It is not well understood if ovarian deficits following neonatal exposure to endocrine disruptors result from direct effects in the ovary itself, disrupted organization of the hypothalamus, both of these mechanisms, or some other potential mechanism, perhaps at the level of the pituitary. Although the present experiments were not designed to explore this critical knowledge gap directly, we again compared the effects of PPT and BPA as a first step in the exploration of this key issue. A recent study by Nakamura and colleagues reported that ovaries collected from mice neonatally exposed to PPT, but not the ESR2 agonist DPN, at 13-weeks of age lack corpora lutea a condition that is accompanied by persistent estrus [30]. This finding suggests that ESR1 may have an important mechanistic role in endocrine disruption of ovarian function. We have also reported persistent estrus in female rats following neonatal exposure to PPT [11] but did not explore ovarian morphology in that prior study. Thus, in the present study, we sought to determine if neonatal exposure to BPA or PPT induces similar ovarian malformations.

Multiple laboratories have now demonstrated that BPA can impair female reproductive function at doses equivalent to or below those that regulatory bodies have deemed to be “safe” for human exposure, but the question of whether or not there are significant “low dose effects” of BPA remains controversial. At the time the present studies were undertaken, the lowest observed adverse effect level (LOAEL) for BPA established by the United States Environmental Protection Agency (EPA) was 50 mg/kg bw per day and the EPA reference dose (the dose considered “safe” for human exposure) was 50 µg/kg bw per day. For our experiments, we used the EPA LOAEL as our “high dose” and the EPA reference dose as our “low dose.” The synthetic estrogen estradiol benzoate (EB), at a dose sufficient to masculinize the hypothalamus, advance pubertal onset, and prevent the onset of regular estrous cycles, was used as a positive control [31-33].

Animals were exposed to either vehicle, EB, PPT, low dose BPA (50 µg/kg bw), or high dose BPA (50 mg/kg bw) in the first four days of life via subcutaneous injection. Injection has recently been demonstrated to produce equivalent or lower plasma BPA levels in neonatal rodents than oral administration thus making injection an appropriate route of exposure when considering potential effects in humans [34] (See also the Draft National Toxicology Program (NTP) Brief on Bisphenol A found online at http://cerhr.niehs.nih.gov/chemicals/bisphenol/BPADraftBriefVF_04_14_08.pdf). Following neonatal exposure, females were then monitored for changes in reproductive physiology, sexual

receptivity, GnRH activation and ovarian morphology. We hypothesized that BPA would adversely affect all endpoints examined, in a dose dependent manner.

Materials and Methods

Animals and neonatal treatment

Animal care, maintenance and surgery were conducted in accordance with the applicable portions of the Animal Welfare Act and the U.S. Department of Health and Human Services “Guide for the Care and use of Laboratory Animals” and approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee. Female pups were obtained from cross-fostered litters born to timed pregnant Long Evans rats (n = 10, Charles River, NC). Cross-fostering was done to minimize potential litter effects. Each litter contained a mixture of animals (12 maximum), only two of which were genetically related to each other. All dams were individually housed in a humidity and temperature controlled room with a 12h light cycle (lights on from 07:00 to 19:00) at 23°C and 50% average relative humidity at the Biological Resource Facility at NCSU and maintained on a semi-purified, phytoestrogen-free diet ad libitum for the duration of the experiment (AIN-93G, Test Diet, Richmond, IN).

Beginning on the day of birth, the female pups were cross-fostered and subcutaneously (sc) injected with vehicle (0.05 ml), estradiol benzoate (EB, 25 µg, Sigma, St. Louis), 50 µg/kg bw bisphenol-A (Low Dose BPA, Sigma), 50 mg/kg bw bisphenol-A (High Dose BPA), or the ESR1 agonist 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; 1 mg/kg bw, Tocris Biosciences, Ellisville, MS). PPT is a selective agonist for ESR1, with a 400-fold preference for ESR1 and minimal binding to ESR2 [35]. EB was used as a positive control and administered at a dose previously established to masculinize the hypothalamus and eliminate GnRH activation in response to hormone priming [31-33]. All compounds were dissolved in ethanol, and then sesame oil at a ratio of 10% EtOH and 90% oil as we have done previously [11, 36]. The vehicle was also prepared with this ratio. We have found this vehicle to cause less skin irritation than the alternative vehicle, DMSO. The animals (n = 10-12 per group) received injections daily from the day of birth, defined as postnatal day zero (PND 0), through PND 3 (four injections total).

All pups were weaned into same-sex littermate pairs on PND 22, ear tagged, and maintained on a reverse light schedule (lights off from 10:00 to 22:00) for the remainder of the experiment. Upon weaning, a cohort of animals (n = 8-9 per group) were checked daily for day of vaginal opening (DOV). The primary mechanism for pubertal onset remains unclear [37, 38] but in rats DOV is a hallmark of puberty and was therefore used as the physiological indicator of puberty. Monitoring of the estrous cycle by vaginal lavage [39] commenced approximately two weeks after vaginal opening and occurred every 24 hours for four days (longer if needed), every other week for 13 weeks. We opted to sample every other week to reduce handling stress and minimize the potential for inducing a pseudopregnancy. All animals observed to have stopped cycling (defined by the failure to sequentially progress through proestrus, estrus, and diestrus) were resampled (for three to five days) two weeks later to confirm that the failure to cycle was not due to a pseudopregnancy.

Females were ovariectomized (OVX) under isoflurane anesthesia (completed over three weeks beginning on postnatal day 148) and allowed two weeks to recover to provide adequate time for endogenous hormone levels to diminish. At the time of OVX, ovaries were removed and fixed in 10% formalin for 24 hours at 4°C, then post-fixed in 70% ethanol and stored at 4°C until processing.

Testing for Sexual Receptivity

To induce sexual receptivity and test for lordosis behavior, the OVX females were sc injected with 10 µg EB at 09:00, followed 48 hours later by a sc injection of 500 µg progesterone (P) (same vehicle as above) and paired with vigorous males as described previously [40-42]. Lordosis quotient (LQ) was calculated by dividing the number of lordosis responses in each trial (10 min) by the number of mount attempts then

multiplying the result by 100. In two pairings, the male made no attempt to mount the female. These trials were not included in the analysis.

Ovarian histology

Ovaries were coded at collection, and subsequently processed at the National Institute of Environmental Health Sciences (NIEHS). For each animal, both ovaries were paraffin embedded side by side, and sliced into 6 μm sections. Sections were slide mounted (Superfrost Plus, Fisher, Pittsburgh, PA) and two slides per animal were deparaffinized, stained with H&E, coverslipped, and qualitatively examined for histological abnormalities at NIEHS. The slides and the remaining uncut tissue were then returned to NCSU for the quantification of corpora lutea (CLs). CL counts are a reliable indicator of successful ovulation [24]. Although the morphological appearance of CLs changes across the cycle, the overall number does not [43], eliminating the need to control for cycle stage at OVX. For each animal, CLs were counted on two slides (each containing one section from each ovary) and averaged to yield a final value for statistical analysis.

Brain collection, and immunohistochemistry

Nineteen days after the behavioral tests were completed, all animals were again sequentially administered EB and P and sacrificed by transcardial perfusion with 4% paraformaldehyde 6-8 hours after the progesterone injection as we have done in past experiments [11]. It is well established that this is the point at which GnRH activity, following sequential steroid hormone administration, is maximal [44, 45]. Brains were removed, post-fixed, cryoprotected, and stored at -80°C (14), then sliced into 35 μm coronal sections, and divided into four series of free-floating alternating sections, one of which was used for the present study. For each animal, one set of coronal sections comprising the organum vasculosum of the lamina terminalis (OVLT) through the caudal border of the anterior ventral periventricular nucleus (AVPV) were immunolabeled for GnRH and FOS using immunohistochemistry (IHC) methods described in detail elsewhere [11, 46]. GnRH and FOS were detected using a cocktail of primary antibodies directed against GnRH (raised in rabbit, 1:20,000, generously gifted by Dr. Robert Benoit, (McGill University Health Center, Montreal, Canada) and FOS (raised in goat, 1:250, Santa Cruz, SC-52-6) followed by the secondary antibodies Alexa-Fluor donkey anti-rabbit 488 and Alexa-Fluor donkey anti-goat 568, each at 1:200. After secondary antibody incubation, sections were rinsed, mounted onto slides (Superfrost Plus, Fisher, Pittsburgh, PA), and coverslipped using a glycerol based mountant (50% glycerol in 4M sodium bicarbonate).

Quantification of GnRH and FOS immunoreactivity

GnRH/FOS double-immunofluorescent label was visualized and quantified as described in our prior publications [11, 47]. Briefly, GnRH and FOS immunostaining was observed to be consistent and distributed both laterally and dorsally to the third ventricle throughout the OVLT, as has been described previously [48, 49]. Thus, 2-3 midlevel sections per animal were selected for analysis. Only those sections showing consistent GnRH and FOS immunostaining were used. They were photographed with a Retiga 1800 monochrome camera attached to a Leica 5000DM microscope fitted with 20X, and 40X objective lenses and filter cubes for Cy3 and FITC. Anatomical identification was made using a brain atlas [50]. The images of each label (GnRH and FOS) were then merged using the MCID Elite Image Analysis (Interfocus Imaging Ltd., Cambridge, England) software package. Cells immunostained for GnRH only, and cells immunolabeled for both GnRH and FOS were then hand counted by an individual blind to the treatment groups and verified by a second independent observer.

Statistical Analysis

DOV, LQ, CL number and percentage of GnRH and FOS co-labeled cells, were compared across treatment groups by one-way analysis of variance (ANOVA) with treatment as a factor and followed up with Fisher's Least Significant Difference (LSD) post-hoc tests for individual comparisons (SYSTAT, Systat Software, Inc., Chicago, IL). As anticipated [48, 49] the number of GnRH neurons counted did not

significantly differ between groups (data not shown). In all cases, comparisons were two-tailed and the significance level was set at $P \leq 0.05$.

Results

Age at Vaginal Opening

There was a significant effect of treatment on DOV ($F(5,50) = 18.34$, $P \leq 0.001$). Compared to the oil treated controls ($n = 9$), DOV was significantly advanced by neonatal exposure to EB ($n = 11$, $P \leq 0.001$), PPT ($n = 8$, $P \leq 0.04$) and the low dose of BPA ($50 \mu\text{g}/\text{kg}$ bw, $n = 8$, $P \leq 0.01$, Fig 1). The high dose of BPA ($50 \text{ mg}/\text{kg}$ bw, $n=14$), however, had no significant effect on DOV.

Estrous Cycle Data

Beginning approximately two weeks after DOV, regularity of the estrous cycle was assessed every 24 hours for four days (longer if needed), every other week for 13 weeks by vaginal lavage in a cohort of animals. Regular 4 day estrous cycles commenced in all treatment groups except the group neonatally exposed to EB (Fig 2). As expected [31-33] all of the EB treated females ($n = 8$) displayed persistent estrus or diestrus and failed to enter a normal estrous cycle. By seven weeks after DOV all ($n= 8$) neonatally treated PPT females had entered a state of persistent estrus or diestrus. By 15 weeks after DOV only 33% of the females exposed to the high dose of BPA ($n = 9$) were still cycling compared to 86% of the females exposed to the low dose of BPA ($n = 8$).

Sexual Behavior

Sexual receptivity was assessed following ovariectomy and hormone replacement. There was a significant main effect of treatment on LQ (Fig 3, $F(4,35) = 17.046$, $P \leq 0.001$), however this effect was exclusively due to the abolition of sexual behavior in the group neonatally treated with EB ($n = 9$, $P \leq 0.001$). There was a trend for suppressed LQ in the group neonatally exposed to low dose BPA but this effect did not reach statistical significance ($n = 7$, $P = 0.157$). Both the PPT and high dose BPA exposures had no appreciable effect on LQ. These observations are consistent with what we have observed in past studies [42].

Ovarian Morphology

Both a qualitative and quantitative analysis of ovarian morphology was conducted (Table 1). Qualitative histological comparison between groups revealed that only the negative control group showed consistently healthy ovaries characterized by all stages of follicular development and the presence of numerous healthy corpora lutea (CLs) (Fig 4). All other groups displayed some degree of abnormalities including hemorrhagic follicles, large antral-like follicles that did not seem to support ovulation, multi-oocyte follicles, and ovarian cysts. The type and severity of abnormalities present differed between treatment groups (Table 1). Females neonatally exposed to EB as our positive control group (Fig 4) displayed the most severe effects, qualitatively characterized by undersized ovaries, no CLs, and no signs of folliculogenesis. Females neonatally exposed to the ESR1 selective agonist PPT also displayed abnormal ovarian development but not in the same way that the EB females did. Ovaries from PPT treated females (Fig 4) were also undersized and lacked CLs but were characterized by numerous large, antral-like follicles that did not appear to support ovulation. Some contained oocytes (Fig 4, yellow arrows) but most did not. Females neonatally exposed to the high dose of BPA ($50 \text{ mg}/\text{kg}$) displayed abnormal folliculogenesis and follicle degeneration with a few follicles containing multinucleated cells. Ovaries from females exposed to the low dose of BPA ($50 \mu\text{g}/\text{kg}$) displayed all stages of follicular development; however a few animals had hemorrhagic follicles. Ovaries from both BPA treated groups also contained a number of large antral-like follicles that were similar in appearance to those seen in the PPT treated animals and structurally consistent with ovarian cysts. All of the PPT treated animals and most of the high dose BPA treated animals were acyclic at the time of ovariectomy, and CLs were not typically observed in these animals. Thus it is unlikely that these follicles progressed to ovulation.

Because the number of antral follicles changes across the estrus cycle and we did not stage our animals at OVX, we did not compare the mean number of antral-like follicles across treatment groups.

Quantitative analysis of ovulatory capacity was made by counting CLs (Table 1). CLs appear following the release of an egg from the ovary and are a reliable and quantitative indicator of successful ovulation regardless of cycle stage [24, 43, 51]. There was a significant treatment effect on the number of CLs present ($F(4,35) = 22.484$, $P \leq 0.001$). Compared to the control females, a significant decrease in CL number was observed in the females neonatally exposed to EB ($n = 8$, $P < 0.001$), or PPT ($n = 7$, $P < 0.001$), neither of which displayed any CLs. The number of CLs was also significantly lower in the high dose BPA treated females ($n = 10$, $P < 0.001$), with only three of ten containing any CLs. The mean number of CLs also trended lower in the low dose treated BPA females compared to the control group but this effect did not reach statistical significance ($n = 8$, $P < 0.36$). This was largely because, while most animals in the low dose BPA group had fewer CLs than the vehicle treated control animals, two animals had nearly twice as many. Both of these animals were still cyclic, but not staged, at the time of ovariectomy.

Co-Localization of GnRH and FOS in the OVLT

Hormone treatment was repeated approximately two weeks after sexual testing was completed, and animals were sacrificed six hours after the P injection. This is the point at which FOS expression in GnRH neurons (an indicator of GnRH activation) is known to be maximal [44, 45]. GnRH activation was assessed by quantifying the percentage of GnRH and FOS colabeled OVLT neurons. There was a significant effect of treatment ($F(4,29) = 17.331$, $P \leq 0.001$). Hormone administration successfully induced FOS immunoreactivity (-ir) in 93% of GnRH neurons of the oil treated control females ($n = 7$, Fig 5). In contrast, 6% of GnRH neurons in EB treated females ($n = 5$, $P < 0.001$), and only 22% in PPT treated females ($n = 7$, $P < 0.001$) were immunoreactive for FOS. This PPT effect is consistent with what we have reported in past studies [11]. FOS labeling within GnRH neurons was not significantly reduced in either of the BPA groups compared to the controls suggesting that the sex specific organization of the hypothalamus may not have been disrupted by neonatal BPA exposure at the doses used.

Discussion

The accelerated pubertal timing, premature anestrus, and ovarian malformations induced by neonatal exposure to BPA could result from disrupted organization anywhere within the HPG axis, including the hypothalamus, ovary, and/or pituitary gland. The exposure was specifically timed to target the hypothalamus. Our results do not support the hypothesis that BPA can defeminize the hypothalamus, a process in which, as we have previously shown [11], ESR1 appears to play a significant mechanistic role. Instead, GnRH neurons seem to retain the capacity to respond (by producing FOS) to hormone priming by estrogen and progesterone. These results suggest that steroid positive feedback was not defeminized by BPA, but do not rule out the possibility, however, that other signaling mechanisms on GnRH neurons could have been altered.

Effects through ESR1 (by PPT or other endocrine disruptors) on GnRH are most likely indirect as ESR1 is not known to be present in GnRH neurons. Instead, disruption likely occurs within ESR1-containing neurons in the AVPV, the arcuate nucleus (ARC) or elsewhere, which send projections to GnRH neurons [23]. This may include the recently discovered population of neurons in the AVPV that express the peptide kisspeptin and send afferents to GnRH neurons [15, 16]. The vast majority of kisspeptin neurons in this region co-express ESR1 and the administration of kisspeptin can induce early puberty in adolescent animals and generate a GnRH surge in ovariectomized animals [52, 53]. A recent study by Navarro and colleagues found reductions in hypothalamic Kiss1 mRNA levels following neonatal BPA exposure at doses higher than those used here [54]. We subsequently determined that the density of hypothalamic kisspeptin-ir fibers is reduced by the high, but not the low dose of BPA used in the present study, and that the effect was more pronounced in the ARC than the AVPV [42]. Collectively, these

observations support the hypothesis that signaling pathways on GnRH neurons may be altered by developmental exposure to BPA and that steroid negative (rather than positive) feedback may be impaired.

Even if GnRH release following estrogen administration is normal, the subsequent release of LH from the anterior pituitary may not be. A new study by Fernandez and colleagues targeted the critical period for pituitary differentiation (PND 13) in female rats. Levels of GnRH induced LH released from pituitary cells, *in vivo* and *in vitro*, were lower than controls in BPA treated animals. Additionally, BPA treated animals showed increased GnRH pulse frequency, hypothesized to cause desensitization of the pituitary thus leading to blunted LH secretion [55, 56]. Prepubertal exposure to BPA has also been shown to decrease LH pulse frequency and amplitude in gonadally intact ewes [57, 58]. Similarly, perinatal but not adult exposure to 1.2 mg/kg bw BPA decreased plasma LH levels in adult OVX mice, an observation which also indicates possible interference with hypothalamic organization and emphasizes that exposure sensitivity is heightened during critical developmental periods. BPA has also been shown to reduce ERK activation [55] which may in turn affect GnRH receptor expression [59]. Reduced GnRH receptor expression in the pituitary could be a mechanism by which LH release is reduced while steroid induced GnRH activation remains intact. Collectively, these prior results combined with the abnormalities seen in the present study could also be indicative of disrupted steroid negative feedback.

Although it is still unclear by what mechanism it might occur, our results clearly demonstrate that ovarian cyclicity is compromised by a very brief, neonatal BPA exposure at doses at and above the current EPA's reference dose for human exposure. Prior studies examining if BPA can affect ovarian function have used equivalent or higher doses than those used here, and shown that the window of vulnerability may extend beyond the neonatal period [55, 60, 61]. For example, exposure to 10 mg/kg BPA on PND 15-18, 100 µg/kg BPA across PND 1-5 or 50 mg/kg BPA on gestational days 6-21 have all been shown to result in ovarian and vaginal malformations in mice [62-64] including the absence of CLs, cystic ovaries, endometrial hyperplasia and lack of ESR1 expression in the vagina. Higher doses of BPA (1 or 4 mg/pup) have also been reported to decrease ovarian area occupied by CLs and multiple cystic follicles in rats [65]. Within that study, however, a dose of 250 µg per pup was not found to alter ovarian morphology, a finding which is inconsistent with our own, but the ovaries were collected approximately six weeks after vaginal opening (earlier than was done in the present study) so it is possible that abnormalities following low dose exposure could have manifested later. Although, in the present study, the low dose of BPA accelerated pubertal onset, most of these animals maintained a regular estrous cycle for 15 weeks after DOV, while animals exposed to the high dose did not. Our results indicate that lower doses of neonatal BPA exposure may result in reduced ovulatory capacity, although the onset of irregular cycles may take somewhat longer to manifest. This type of effect has also been observed following neonatal low dose exposure to the phytoestrogen GEN [24]. Therefore it is important to observe animals for several weeks beyond pubertal onset when seeking to determine if an EDC can affect ovarian function. The effect of low dose BPA on ovarian cyclicity may be more robust if exposure comprises both the gestational and neonatal periods, a condition that is more consistent with human exposure, which is low and life-long [6].

It has been hypothesized that neonatal exposure to EDCs enhances ovulation rate when animals are young, which then results in compromised fertility later in life and induces a premature reproductive senescence [24]. The intriguing observation that two females exposed to the low (50 µg/kg bw) dose of BPA in the present study had nearly twice as many CLs as control females while the rest had fewer is consistent with this hypothesis and requires further investigation. It is possible that, among these animals, CL counts were different simply because they were at different points in their estrous cycle when the ovaries were collected. A recent study, however, found that although the morphologic features of CLs change across the cycle, the number of CLs does not [43].

Our results also indicate that lower rather than higher doses of BPA have the greatest potential to accelerate pubertal onset, a finding which is counterintuitive but consistent with what has been seen in other studies. An even lower dose (20 $\mu\text{g}/\text{kg}$) administered over gestational days 11-17 has also been shown to advance vaginal opening [66], demonstrating that perturbations of the hormonal milieu in other critical developmental windows besides the neonatal period have the potential to advance puberty. Conversely, higher doses of BPA typically fail to affect pubertal timing, or delay rather than advance it [63]. The mechanism by which lower, but not higher, doses of BPA can advance puberty remains poorly understood. It has previously been hypothesized that BPA, like hormones, has an inverted U-shaped or “nonmonotonic” dose response curve [8, 67-69]. Our results are consistent with that hypothesis. The mechanism(s) by which this might occur have not been satisfactorily elucidated but could include stimulation of hormone receptors at low doses but down-regulation of hormone receptors at higher doses [For a critical discussion of nonmonotonic dose curves see ref 70]. It is important to note that, although the effect of neonatal exposure to the low dose of BPA on DOV was significant, it was not as robust as neonatal exposure to EB. Neonatal exposure to the ESR1 agonist PPT was also not sufficient to advance pubertal onset to the same degree as EB. This could suggest that strong agonism of both ESR1 and ESR2 is necessary to evoke a maximal response.

It is also possible that BPA alters pubertal timing by an ER independent mechanism. For example, body weight at the time of vaginal opening may influence this endpoint and could therefore be a confounding factor. Delayed pubertal onset following exposure to 500 mg/kg BPA via maternal dietary exposure during gestation and nursing, was most pronounced in the lightest animals [71]. Other studies in rats and sheep have also found an effect of BPA on body weight at pubertal onset [58, 60]. Therefore, it could be that vaginal opening in our high dose (50 mg/kg) animals was later than in our low dose (50 $\mu\text{g}/\text{kg}$) animals because of a difference in body weight. Unfortunately, this possibility was not taken into account, however we did not note any obvious differences in body weight at the time of vaginal opening. Regardless, our data suggest that exposure to BPA during a developmental window that corresponds to late gestation in humans, at levels relevant to human exposure, has the potential to accelerate pubertal onset. The specific mechanisms by which this occurs remain to be determined.

Malformations in the ovary following exposure to EDCs, including BPA, during development have been reported previously, but the mechanism(s) by which this might occur has not been well established [9]. For this aspect of the present study, the ESR1 selective agonist PPT was employed for comparative purposes. All groups except the control group showed some degree of abnormalities, the characteristics of which were distinctly different between groups. Malformations within the BPA females were dose dependent, with ovaries from the high dose group showing the most significant adverse effects. Generally, the BPA ovaries more closely resembled those from the PPT than the EB group which, as expected from prior reports [72], were notably undersized (a qualitative assessment) and showed no sign of folliculogenesis. Unlike the EB group, ovaries from animals in the PPT and high dose BPA groups were characterized by numerous antral-like cavities, most of which contained either a degenerating or no oocyte. In many cases, these cavities resembled cystic follicles but some appeared to be large atretic follicles, particularly in the PPT animals (Fig 4). This observation is consistent with a previous study which also described the presence of large, antral-like cavities in the ovaries of mice exposed to 250 $\mu\text{g}/\text{kg}$ bw BPA in utero [73] and to a more recent study [74] which observed similar structures in cultured follicles directly exposed to BPA. It should be noted, however, that a different mechanism of action is likely involved in the cell culture study because the ovarian malformations arose from acute rather than developmental exposure. Finally, none of the PPT females had CLs, an observation which is consistent with what has been previously reported in mice [30] and suggests that the PPT animals are anovulatory and fail to progress beyond the follicular phase. It appears that agonism of ESR1 during rodent neonatal period can curtail the capacity of oogenesis to progress into the luteal phase.

Although the ovaries from the animals neonatally treated with PPT and the high dose of BPA share some similar characteristics, they do not necessarily indicate a mechanistic role for ESR1 in the emergence of the BPA induced effects. Moreover, a mechanistic role for ESR2 in the disruption of ovarian function by BPA cannot be ruled out. Recent studies have reported that ESR2 deficient mice, exposed gestationally to 400 ng/day of BPA do not display the same abnormalities as BPA-exposed wild type controls [75] leading the authors to conclude that BPA exerts its effects in the ovary via ESR2 [75, 76]. It is important to be mindful, however, that effects resulting from the elimination of ESR2 function across the lifespan may differ from those observed after selectively agonizing or silencing ESR2 during discrete time periods. We and others have shown that postnatal exposure to ESR2 specific agonists can also result in the premature loss of a regular estrous cycle, although not necessarily at the same rate as animals exposed to ESR1 specific agonists [11, 77]. Therefore the loss of ovarian cyclicality appears to be possible following postnatal agonism of either of the two major ER subtypes. ESR1 is primarily expressed in the interstitial and thecal cells as early as PND 1 while ESR2, expressed in the granulosa cells, is not detectable on PND 1 but present by PND 5 [78]. Thus, the BPA exposure in our study was sufficiently long enough for either ER subtype to play a mechanistic role. It is also possible that BPA is acting as an ESR 1 or ESR2 antagonist. Selective antagonists for ESR1 and ESR2 are only now becoming available so it may soon be possible to directly test how ER specific antagonism affects ovarian structure and function as part of future studies. These and other types of experiments are needed delineate the relative roles each ER subtype play in the organization and function of the HPG axis including the ovary.

It is also possible that BPA action on ovarian development and HPG organization may not be limited to ERs. BPA is thought to bind to the thyroid hormone receptor, androgen receptor, estrogen-related receptor and the aryl hydrocarbon receptor [70, 79-81] and may have direct or indirect actions on the ovary through these alternate pathways. For example, estrogen related receptor gamma (ESRRG) and estrogen related receptor alpha (ESRRA) expression have been found in human ovarian cells with increased expression in ovarian cancer cells [82, 83]. BPA can also bind to a membrane-bound form of the ER and a transmembrane ER called G protein-coupled receptor 30 (GPER, formerly called GPR30) [84-86]. Emerging evidence now indicates that epigenetic modifications such as DNA methylation of transposable elements and cis-acting, imprinting regulatory elements, may also be a potential mechanism by which BPA and other endocrine disruptors affect reproductive function [87, 88]. The potential for BPA and other endocrine disruptors to modify the epigenome, and the health consequences of those disruptions remains largely unexplored.

Interestingly, when hormone primed following ovariectomy, all of the animals, except those treated with EB, displayed normal sexual receptivity, regardless of whether or not they had lost their estrous cycle, demonstrating that the capacity to display sexual behavior may persist despite impaired reproductive physiology. This observation is consistent with what we have seen before following neonatal exposure to GEN, EQ, PPT, or DPN [11, 42]. Enhanced lordosis has previously been reported following gestational exposure to 40 µg/kg/day of BPA [89] or a low (5 mg/kg) dose of the chlorinated pesticide chlordecone, a compound which is also classified as an estrogenic EDC [90]. Our observations are also consistent with a body of literature produced by Gorski and others showing that lordosis behavior can persist following postnatal hormone manipulation depending on dose and timing of administration [91-93]. We have yet to determine whether or not these females would display normal sexual behavior if tested with ovaries in place and without hormone priming.

Here we have shown that neonatal exposure to BPA at or below the LOAEL set by the EPA adversely affects pubertal timing and ovarian function. Our results are consistent with the conclusions of the NTP's Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A [94] (<http://cerhr.niehs.nih.gov/chemicals/bisphenol/bisphenol.pdf>) which stated that there is "some concern for adverse effects of developmental toxicity for fetuses, infants and children." The NTP also concluded that there is "minimal" concern for effects on puberty in females largely because the mouse literature on

this effect is inconsistent and not enough information has been obtained from rat studies. Therefore our study adds to the literature demonstrating an effect on sexual maturation in females. BPA is known to cross the placenta and pass to infants by lactational transfer [5, 95, 96]. Blood and amniotic fluid samples obtained from pregnant women reveal that amniotic levels of BPA (8.3-8.7 ng/ml) can be even higher than fetal serum levels (1-2 ng/ml) [97-99]. Whether or not the levels of BPA obtained through maternal exposure in humans are sufficient to elicit reproductive deficits remains to be determined. However, as contamination levels and routes of exposure to both naturally occurring and synthetic endocrine disrupting compounds differ across populations, determining exactly how they interfere with normal reproductive development warrants further exploration. Our finding that the capacity for GnRH neurons to produce FOS in response to hormone priming is apparently intact in female rats neonatally exposed to BPA suggests that defeminization of the hypothalamus may not be a mechanism by which developmental exposure to BPA can affect DOV, estrous cyclicity, and ovarian morphology. Further studies are needed to better characterize the specific mechanisms through which BPA, and similar EDCs, disrupt the organization of the HPG axis and impair female reproduction.

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Figure Legends

Fig 1. Day of vaginal opening (DOV) was significantly advanced by neonatal (PND 0-3) administration of estradiol benzoate (EB) and the low (50 µg/kg bw) dose of bisphenol-A (BPA) but not the high (50 mg/kg bw) dose of BPA or the ESR1 selective agonist PPT compared to the control animals. (Means ± s.e.m., *P ≤ 0.04)

Fig 2. Regular estrous cycles commenced in all groups except the group neonatally exposed to EB. The percentage of females within each treatment group displaying a regular estrous cycle diminished over time in all groups except for the control group. By 15 weeks after vaginal opening, only 33% of animals exposed to the low (50 µg/kg bw) dose of BPA neonatally still displayed a regular estrous cycle compared to 86% of the animals exposed to the high (50 mg/kg bw) dose of BPA.

Fig 3. Female sexual receptivity, following hormone priming, was not significantly affected by neonatal exposure to any of the compounds except for EB (Means ± s.e.m., *P ≤ 0.001, LQ = Lordosis Quotient)

Fig 4. Ovarian morphology was distinctly different across treatment groups. Females neonatally exposed to EB had very small ovaries with no clear signs of active oogenesis or ovulation. Ovaries from females neonatally exposed to PPT were characterized by numerous large antral-like follicles, only some of which contained oocytes (yellow arrows). Similar structures were observed in both of the BPA treated groups but not to the degree seen in females neonatally exposed to PPT. Generally, ovaries from the high (50 mg/kg bw) dose BPA treatment group more closely resembled those from the PPT treatment group than the EB treatment group. (scale bar = 500 µm)

Fig 5. (A) Immunolabeling of GnRH (red) and FOS (green) in the OVLT. Double labeled cells are indicated by the white arrows and single labeled GnRH neurons are indicated by the blue arrows. The number of GnRH immunopositive cells did not significantly differ among groups. The percentage of GnRH cells that were colabeled with FOS was significantly lower in animals neonatally exposed to PPT or EB but not significantly affected by neonatal exposure to either the low (50 µg/kg bw) or high (50 mg/kg bw) dose of BPA. (3v = 3rd ventricle, Means ± s.e.m., P ≤ 0.001, *P ≤ 0.001, scale bar = 40 µm)

Table 1. Summary of ovarian morphology.

Treatment Group	Corpora Lutea animals with CLs/ total 'n' in group (quantitative)	Mean Number of CLs present	Ovary Size (qualitative)	All Stages of Folliculogenesis Present?	Characterization of Folliculogenesis	Appears Likely to Support Ovulation (qualitative)
Control n = 8	8/8	12.43	Normal	Yes	Appears normal	Yes
EB (25µg) n = 8	0/8	0	Undersized	No	Abnormal: small follicles	No
PPT (1 mg/kg) n = 7	0/7	0	Undersized	No	Abnormal: many larger antral and cystic follicles, degenerating eggs	No
BPA (50 µg/kg) n = 7	7/7	9.75	Slightly Undersized	Yes	Somewhat Abnormal: hemorrhagic follicles in some	Yes
BPA (50 mg/kg) n = 11	4/11	2.1	Normal	No	Abnormal: degenerated, multinucleated, some hemorrhagic tissue present	No

Figure 1

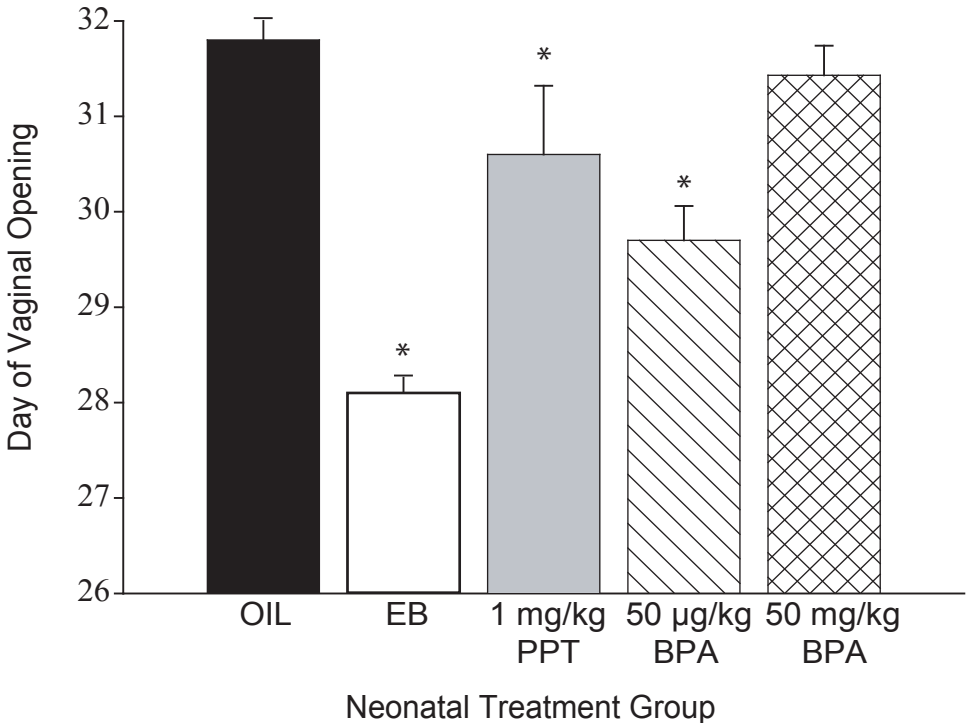


Figure 2

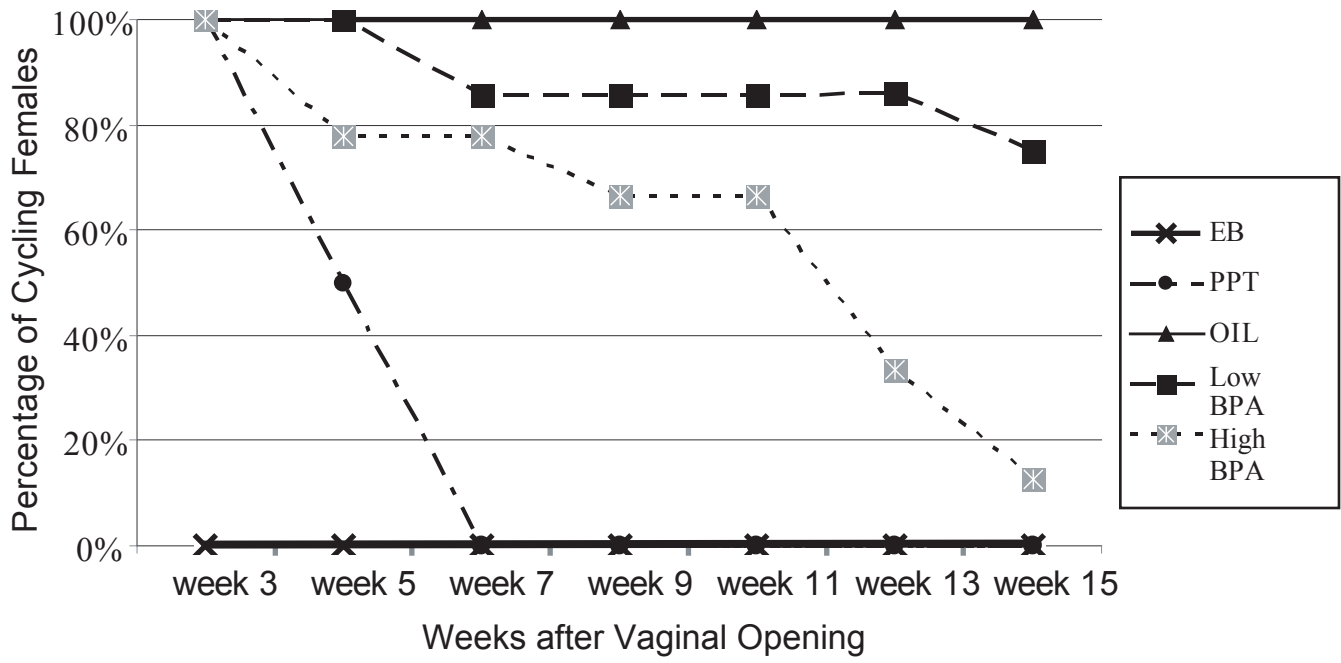
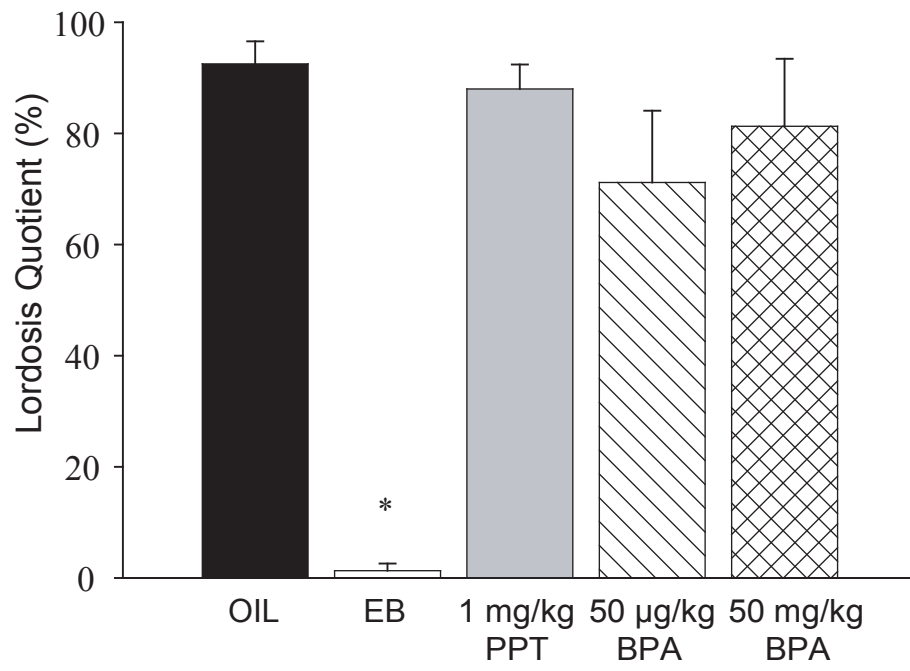
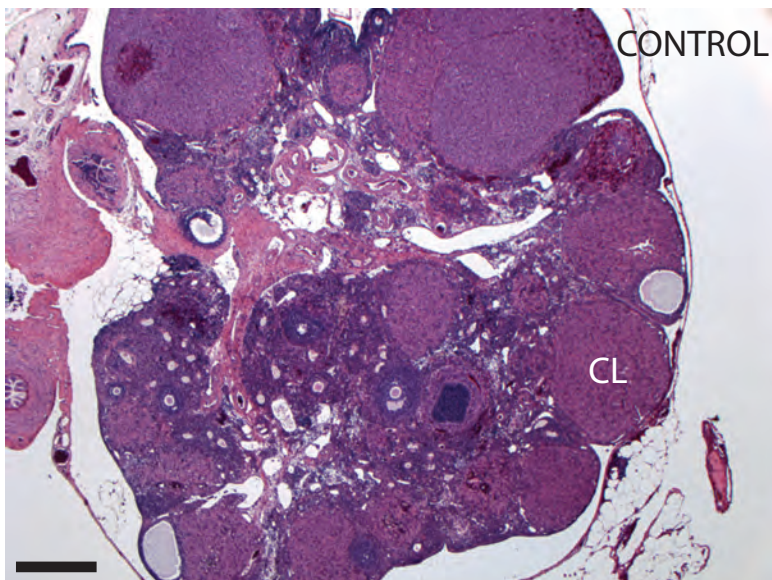


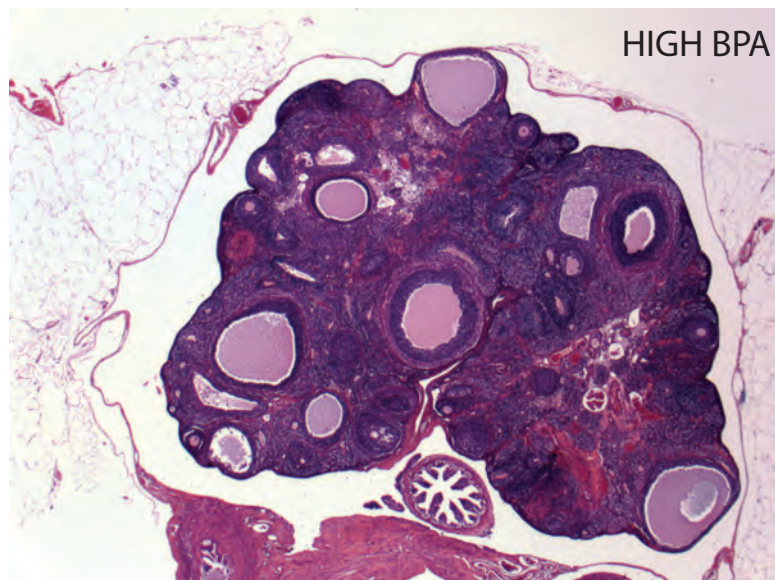
Figure 3



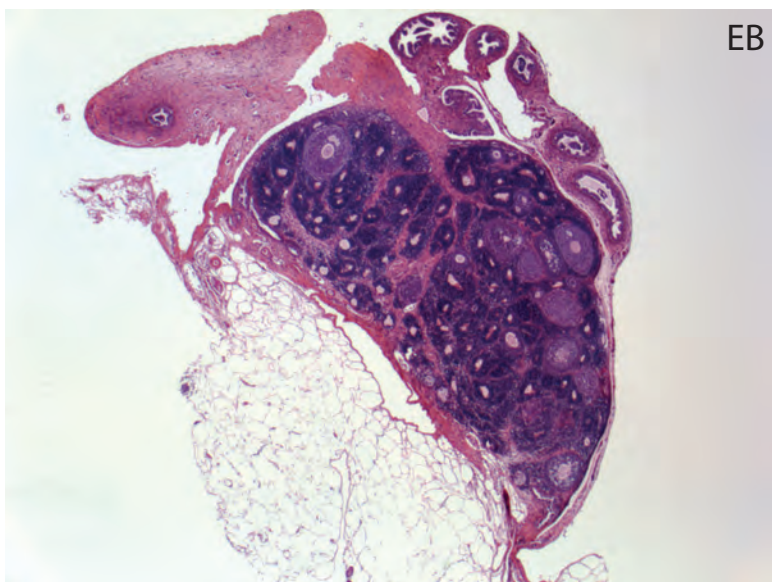


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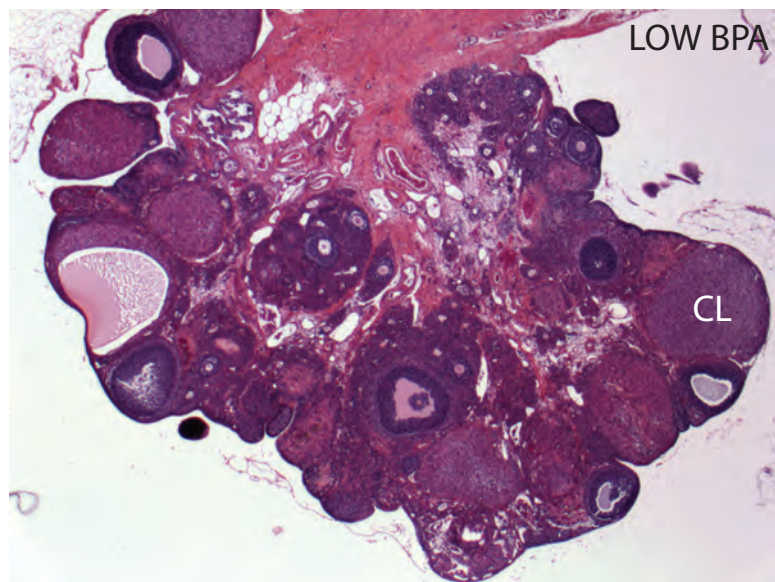
CL



HIGH BPA

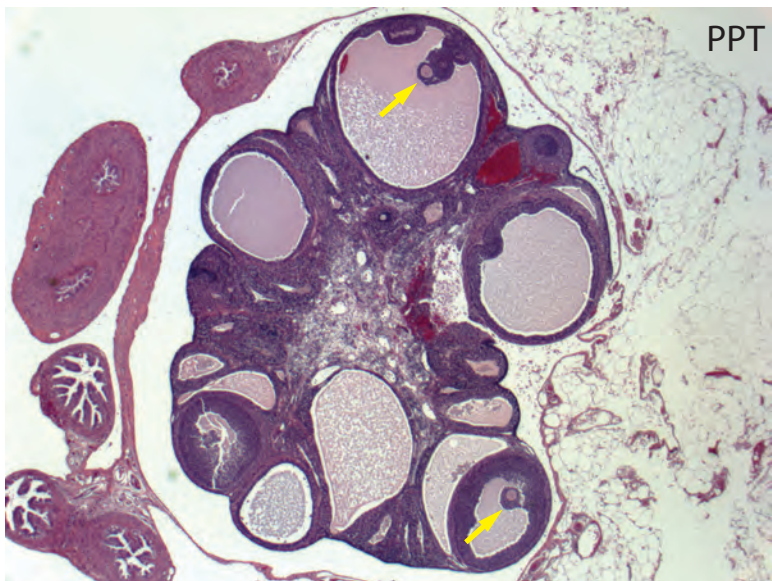


EB



LOW BPA

CL



PPT

figure 5

