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Early Programming of Uterine Tissue by Bisphenol A: Critical Evaluation of Evidence from Animal Exposure Studies

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Abstract

Exposure to Bisphenol A (BPA) during the critical window of uterine development has been proposed to program the uterus for increased disease susceptibility based on well-documented effects of the potent xenoestrogen diethylstilbestrol. To investigate this proposal, we reviewed 37 studies of prenatal and/or perinatal BPA exposure in animal models and evaluated evidence for: molecular signatures of early BPA exposure; the development of adverse uterine health effects; and epigenetic changes linked to long-term dysregulation of uterine gene expression and health effects. We found substantial evidence for adult uterine effects of early BPA exposure. In contrast, experimental support for epigenetic actions of early BPA exposure is very limited, and largely consists of effects on *Hoxa* gene DNA methylation. Critical knowledge gaps were identified, including the need to fully characterize short-term and long-term uterine gene responses, interactions with estrogens and other endogenous hormones, and any long-lasting epigenetic signatures that impact adult disease.

Keywords

endometrium; epigenetic programming; estrus cycle; mouse; rat; uterus; xenoestrogen; endocrine disruptor

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7. Declaration of interest

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1. Introduction

Exogenous exposure to natural hormones and hormone-mimetic chemicals during early life can induce permanent changes in development, and is proposed to increase disease risk during adulthood [1]. This hypothesized developmental origin of adult disease has been widely discussed in relation to the potential long-term effects of exposure to xenoestrogens [2,3] and derives strong support from the reproductive abnormalities seen in female offspring of women exposed to diethylstilbestrol (DES) during pregnancy [4]. *In utero* exposure to DES, a potent estrogenic chemical, was first associated with development of clear cell adenocarcinoma of the cervix in young women [5]. Other abnormalities emerged as DES daughters aged, including decreased fertility, increased rates of ectopic pregnancy, and early menopause [6]. The widespread and persistent exposure of humans and other mammals to other environmental estrogens, including the plasticizer bisphenol A (BPA), is a major public health concern [7,8].

BPA was long considered a weak estrogen, as its binding affinity for the estrogen receptors, ERα and ERβ, was estimated to be 1,000–10,000-fold lower than that of 17β-estradiol [9,10] – in contrast to DES [11]. However, recent studies show that BPA can promote estrogen-like activities with a potency similar to or greater than that of 17β-estradiol, which may reflect alternative estrogenic mechanisms of BPA action [12], including rapid responses via non-classical estrogen signaling pathways [13,14] and differences in co-activator recruitment between BPA and 17β-estradiol [15]. Thus, emerging evidence of the estrogenic activity of BPA at low doses and its high affinity for uterine tissue [16], together with the fact that the developmental health effects of DES in humans are seen over a wide range of exposure doses [17], indicate a clear need to rigorously examine the extent to which BPA shares some of the well documented adverse health effects of DES, including reproductive toxicity [18].

BPA is widely used in the manufacture of polycarbonate plastics and epoxy resins, leading to significant exposure under normal conditions of use via food and beverage storage containers, impact-resistant baby bottles, dental-sealants and composites, and many other materials [19]. Substantial levels of BPA are readily measured in human tissue samples, including serum and urine from children, maternal and fetal plasma, amniotic fluid, and breast milk of nursing mothers [20–22]. BPA acts as a selective ER modulator, but can also bind to other hormone receptors and thereby impact multiple endocrine-regulated pathways [23–26]. BPA crosses the placental barrier [27] and has been linked to adverse human reproductive effects, including recurrent miscarriage [28,29]. In animal models, perinatal exposure to low, environmentally relevant doses of BPA induces developmental defects in brain function and behavior, the male reproductive system, and the mammary gland [30–32], where an increased predisposition to cancer has been observed [33]. BPA exposure is associated with several reproductive toxicities, as seen in animal models and/or in women. Thus, BPA affects meiosis in ovaries, accelerates follicle transition, reduces oocyte quality in animal models and in women undergoing in vitro fertilization, impairs uterine endometrial proliferation, decreases uterine receptivity, and increases implantation failure [18,34]. Given the prevalence of BPA exposure, as well as the long term deleterious effects of early DES exposure in the female reproductive tract, there is much interest in

understanding the impact of early exposure to BPA on uterine tissue and any changes in developmental programming that may lead to adult-onset disease.

BPA and certain other environmental chemicals can disrupt the programming of cells and tissues by epigenetic mechanisms that induce long-term changes in chromatin structure and gene expression [31]. BPA-induced programming may involve several interrelated epigenetic mechanisms, including changes in DNA CpG methylation, alterations in histone modifications, and dysregulation of noncoding RNA expression [35]. Methylation of genomic DNA and/or covalent modification of the core histones that package DNA into nucleosomes, e.g., *via* histone methylation, acetylation, ubiquitylation, and sumoylation, may alter gene expression by changing chromatin packaging density and the accessibility of DNA for transcription factor binding. Short noncoding RNAs can silence genes by mRNA degradation, translation arrest, and miRNA-dependent chromatin remodeling, while long, intergenic noncoding RNAs (lincRNAs) can serve as molecular scaffolds that direct histone modifying enzymes to specific genomic loci [35–37].

There is increasing evidence in non-uterine tissues that BPA can introduce epigenetic changes involving one or more of the above mechanisms, with DNA methylation being the most frequently analyzed epigenetic outcome [31]. These studies cover multiple species, tissue/cell types, exposure paradigms and analyzed outcomes. However, the mosaic of reported results does not provide a clear and consistent mechanistic understanding of the epigenetic effects of BPA exposure. Examples of findings include increased DNA methylation of genes associated with tumor development in human mammary epithelial cells exposed to BPA [38] and the induction of several thousand differentially methylated genomic regions (DMRs) in PND21 rat mammary tissue following prenatal BPA exposure [39]. Further, BPA increased histone methyltransferase EZH2 and its repressive histone-H3 lysine-27 trimethylation marks, in MCF-7 breast cancer cells and in mammary tissue of mice exposed to BPA *in utero* [40]. In other studies, BPA exposure of rats during gestation and lactation altered glucose and insulin tolerance in F2 offspring in association with increased DNA methylation of the *Gck* gene in F2 liver, but with a decreased global level of DNA methylation in F1 sperm [41]. In testis of adult mice exposed to BPA neonatally, DNA methyltransferases *Dnmt3a* and *Dnmt3b* were up regulated and the promoters of *Esr1* and *Esr2* (which encode estrogen receptors ERα and ERβ) became hypermethylated [42]. *In utero* exposure to BPA induced dose-dependent changes in expression of *Dnmt1* and *Dnmt3a*, as well as *Esr1* and *Esr2*, in mouse brain, in a manner that is sex-dependent and region-specific [43]. *Esr1* gene DNA methylation was significantly increased in male prefrontal cortex and was decreased in the hypothalamus of females in association with disruption of sexually dimorphic social and anxiety-related behavior [43]. Further, the methylation status of *NotI* loci in mouse forebrain was altered, with some gene regions showing increased methylation and others showing decreased methylation following *in utero* BPA exposure [44]. CpG methylation was decreased in an intracisternal A particle retrotransposon (IAP) upstream of the *Agouti* gene [45]. BPA exposure *in utero* also disrupts genomic imprinting, leading to decreased methylation of DMRs and altered gene expression in mouse placenta [46]. Human exposure to BPA has been associated with decreased DNA methylation: BPA-exposed male factory workers in China showed significantly lower levels

of LINE-1 methylation (a marker of genome-wide methylation) than non-exposed workers [47], and in a cohort of Egyptian girls, urinary BPA concentrations correlated with CpG methylation profiles in saliva DNA, with BPA-high individuals (urinary BPA > 2 ng/ml) showing more hypomethylated sites than BPA-low individuals (urinary BPA < 1 ng/ml) [48].

In some cases, epigenetic changes induced by early BPA exposure, and associated changes in gene expression, may not become apparent until the onset of puberty. For example, DNA methylation of *PDE4D4* in rat prostate normally increases at puberty, leading to repression of *PDE4D4* in adult rat prostate. Neonatal exposure to BPA interferes with this pubertal increase in *PDE4D4* methylation, and consequently, *PDE4D4* is hypomethylated and gene expression remains elevated at adulthood [49]. A similar failure to develop a hypermethylated state at puberty was seen for *Nsbp1* in mice exposed to DES or genistein during the neonatal period [50]. These findings support the hypothesis that BPA-induced "early-life epigenetic memories" [51] require a hormonal trigger later in life in order to be manifested phenotypically. Thus, we can anticipate two types of gene responses to an early estrogenic exposure: some genes may undergo early, and long-lasting (permanent) epigenetic changes that are linked to changes in gene expression already detectable prior to puberty; these changes constitute an *early molecular signature* of the exposure. Other genes may show a delayed response, i.e., a change in expression may not be apparent immediately following BPA exposure. In such cases, long-term changes in expression do not occur until such changes are triggered by hormonal events that onset at puberty. Here, we use the terms "programing" and "disrupted programming" to refer to molecular events that BPA activates, and which lead to changes in epigenetic marks, gene expression patterns, and/or (patho)physiology. These effects of BPA programming may or may not be manifested immediately after prenatal, perinatal or neonatal BPA exposure, but persist long after termination of the exposure.

Three key questions arise regarding the impact of early BPA exposure on adult uterine tissue: 1) Does BPA program or otherwise alter uterine development in a manner that increases susceptibility to reproductive dysfunction or disease later in life? 2) Which epigenetic mechanisms underlie this disruption of programming by BPA? 3) What is the best way to identify these programming events and help close the gaps in our understanding of the long-term actions of BPA in the uterus? To address these questions, we made the following assumptions to help distinguish long-term BPA-induced programming from shorter-term effects of BPA, which may include direct toxic effects of the exposure, as well as non-toxic responses that are reversed once the exposure is terminated: 1) long-term programming by BPA is only achieved when the exposure occurs during a critical developmental window; 2) programming involves epigenetic changes that impact gene regulation, and may or may not be associated with a discernable short-term phenotypic (e.g., gene expression) response [52]; 3) epigenetic changes are activated during, or shortly after the initial exposure, persist well beyond termination of the exposure, and lead to the development of pathophysiology later in life, e.g. after puberty, in the case of the uterus and other gonadal hormone-responsive tissues.

Here, we present a systematic review of all available primary research publications that use animal models to investigate the impact of early BPA exposure on the uterus. 37 animal studies of prenatal and/or perinatal BPA exposure were identified and then evaluated for evidence of molecular signatures of early BPA exposure, the development of adverse uterine effects, and the induction of epigenetic changes linked to long-term dysregulation of uterine gene expression and health effects later in life. Seven other studies were excluded from our analysis because of concerns regarding the implementation of proper controls for the effects of estrus cycling on outcomes. Our review of these studies shows that early exposure to BPA induces uterine abnormalities in rodent models during adulthood, and we identify critical knowledge gaps regarding the epigenetic actions of BPA and their impact on our understanding of the mechanisms that contribute to uterine tissue programming by BPA.

2. Methods

A library comprised of 1,006 publications was created and updated as of February 25, 2015 by exporting from PubMed all references containing the key words ‘uterus’ or ‘reproduction’ in combination with ‘BPA’ or ‘bisphenol A’. The following studies were then filtered out from the library: review papers, epidemiological studies, studies that do not employ animal models, studies that do not report outcomes related to the uterus, studies that administer BPA in combination with other chemicals, and studies in which animals were exclusively treated with BPA either prior to or after the period of uterine organogenesis and differentiation. In mice and rats, this period extends from embryonic day 11 (E11) through postnatal day 15 (P15) [53]; accordingly, studies of exposures that did not overlap this developmental period were excluded. A total of 44 of the 1,006 publications met the above criteria. These 44 publications were analyzed with respect to their experimental design. Seven publications in which estrus stage-dependent outcomes were analyzed without proper control of estrus cycle were filtered out. The resulting 37 publications (Table 1) were further analyzed and with respect to the impact of prenatal and/or perinatal BPA exposure on the following: 1) early molecular signatures of the exposure, as determined after conclusion of the exposure, but prior to day P20 (rodent studies); 2) adverse uterine health effects in pubertal and adult animals, as determined after day P20 (rodent studies); and 3) changes in epigenetic programming. The only non-human primate study selected for this review reports early molecular signatures, as gene expression was assessed in *in utero* exposed embryos [54], and the only study of hen model reports uterine effects in adult animals [55]. The 37 studies reviewed report uterus-related outcomes following exposure to BPA over wide range of developmental time periods using a variety of exposure routes and an 8 log-range of BPA doses, making it difficult to draw firm conclusions regarding dose-response relationships from the available datasets. Furthermore, given ongoing controversies regarding how to best estimate current levels of BPA exposure in the general human population [8], we did not analyze these studies in the context of safety and regulatory decisions related to human exposure to BPA.

3. Results

3.1. Evidence for early molecular signature of prenatal and perinatal BPA exposure

We identified 11 studies in which the molecular outcomes of early BPA exposure were studied in animals and tissues collected prior to puberty [54,56–64]. One study was carried out using rhesus macaque [54], and all other studies used laboratory rodents. The time interval between the last exposure to BPA and analysis of the molecular outcome ranged from 2 hr [59] to ~20 days [56,65]. Responses that are measured soon after termination of BPA exposure are presumed to represent short-term, reversible effects of BPA, in the absence of evidence to the contrary, while responses seen at later time points have a greater likelihood of representing early molecular signatures of the exposure that persist and may have pathophysiological consequences later in life.

In one study, the effects of three estrogenic chemicals – BPA, 17α-ethynyl-estradiol, and genistein – were investigated in rat embryonic uteri and ovaries harvested 2 hr after the last exposure exposure on E20 [59]. Gene expression changes were determined by microarray analysis of RNA isolated from pooled uteri and ovaries. A total of 397, 381, and 366 genes were differentially regulated by BPA, genistein and 17α-ethynyl-estradiol, respectively. Sixty six genes were differentially regulated in the same direction by all three chemicals. Overall, the gene expression profiles induced by 17α-ethynyl-estradiol and BPA were more similar to each other than to that induced by genistein. The experimental design, involving analysis of RNA pooled from two distinct tissues - uteri combined with ovaries - is a notable limitation that may decrease sensitivity for detection of gene expression changes specific to one of the tissues. Moreover, given the 2 hr time interval from the last exposure to the time of tissue collection, this study, and others with a similar design, does not address the question of whether early BPA exposure induces long-term effects leading to permanent dysregulation of gene expression.

A second microarray study identified 662 genes that were differentially expressed in rat uterus 1 day after conclusion of high-dose BPA exposure (600 mg/kg/day) from days P14 to P16 [64]. The gene expression changes observed likely represent a short term, reversible response to BPA, given the short (1 day) interval between the last exposure and RNA analysis. In two other studies using the same high daily dose of BPA but different exposure windows, significant increases in uterine expression of estrogen regulated CaBP-9k protein and RNA were observed [58,63]. In one study pups were breast fed by dams exposed to BPA from day P1 to P5 and euthanized 24 hr after the last BPA injection [58], and in the second study pups were exposed to BPA *in utero* from E17 to E19 and euthanized on P5 [63]. No changes in uterine weight, genesis of uterine glands and ERα expression were found on P14 or P21 in offspring of Donryu rats exposed to 0.006 and 6 mg/kg/day from E2 to P21 [62].

Significant decreases in uterine expression of *Hoxa10* and *Hoxa11* were seen on day P8 in rats exposed to BPA at 0.05 or 20 mg/kg/day during the neonatal period (days P1, P3, P5, and P7) [60]. Neonatal BPA exposure also decreased the responsiveness of ovariectomized rats to activation of *Hoxa* and other genes by progesterone + estrogen stimulation at adulthood; similar effects were found for neonatal DES exposure [60,66]. Accordingly, the

effects of BPA on *Hoxa* genes seen on day P8 likely constitute an early molecular signature of BPA exposure (i.e., an early indication of long-term epigenetic programming by BPA), rather than a transient response. The same exposure caused a significant decrease in fertility and fecundity [60,66]. In contrast, others reported that in mouse uterus, Hoxa10 protein levels were up regulated 2 weeks after *in utero* exposure to BPA at doses of 0.5, 1 or 5 mg/kg/day [65], and that methylation of the *Hoxa10* promoter and intronic region was decreased in 2 week old mice exposed to the 5 mg/kg/day dose of BPA [56] (also see below). BPA induction of ER target genes was also seen in neonatal rats exposed to BPA (50 mg/kg/day) from P10 to P12. Uterine transcript levels measured 6 hr after the last BPA exposure were altered for 8 of 18 genes containing known or putative ER response elements [57], consistent with these being short-term, ER-dependent estrogenic responses.

In one study, rhesus macaques were exposed to BPA *in utero* at 0.4 mg/kg/day during either of 2 developmental windows, E50-E100 and E100-E165 [54]. Uteri were harvested on the last day of exposure and genome-wide gene expression was analysed. Many more differentially expressed genes were identified for the exposure ending on E165 (883 genes) compared to E100 (84 genes). Pathway analysis for the genes differentially expressed on E165 identified two highly significant networks: one network included several Hox and Wnt genes and related to post-translational modification, protein degradation, and protein synthesis; and the second network placed estrogen receptor as a hub and included genes of cellular and embryonic development. No histological differences between uteri of exposed and control foetuses, and no differences in immunohistochemical staining for ERa, PR, or levels of the proliferation marker Ki-67 were seen at either time point [54].

3.2. Evidence of delayed response

Thirty-six studies reported uterine effects in adult animals after an early developmental exposure to BPA. Two studies [61,67] reported effects of postnatal BPA exposure on uterine ERα and ERβ levels in young adult (P30) and adult (P70) rats, respectively, but based on the experimental design, the uterine samples were apparently not collected at a consistent point in the estrus cycle. Accelerated puberty (i.e., ~1 day advance in vaginal opening) was observed in young mice exposed to BPA on day P8 at doses ranging from 0.1 to 100 mg/kg, as was a decrease in the number of days spent at estrus, albeit only at the 100 mg/kg BPA dose, based on estrus cycle monitoring over a 9 day period beginning at the onset of puberty [68]. However, these results may not be reliable, as estrus cycling is not yet consistent in mice at this age, and given the small number of individual mice studied (n=5 per group). This study, and three others [69–71], examine effects of BPA on uterine weights. Unfortunately, none of these studies [61,67–71] reported measures to control for effects of the estrus cycle on uterine weights, i.e. by collecting uteri at a consistent stage of the estrus cycle. The lack of control for stage of the estrus cycle is a major limitation, as the uterus undergoes large estrus cycle-dependent changes in size and weight, changes that are associated with increased electrolytes and water imbibition in response to physiological changes in estrogen levels; indeed, these changes serve as the basis of the uterotrophic assay for estrogenic activity. The uterus also responds to estrogens by increasing cell division, which contributes to uterine growth [72,73]. These studies [61,67–71] were therefore excluded from further consideration.

3.2.1. Uterine weight—Seven of the 37 studies that properly controlled for estrus cycle investigated effects on adult uterine weights. These studies found that uterine weights were unaffected in adult rats and mice exposed to BPA using a range of doses and dosing protocols [62,74–79], or were affected only at a very high dose (500 mg/kg/day [75]).

3.2.2. Estrus cycle—Fifteen studies examined changes in uterine cyclicity in adult mice and rats after early developmental exposure to BPA [62,74–76,78–88]. In two multi-generational exposure studies, no effect of continuous BPA exposure was seen in adult Sprague-Dawley rats [75] and CD-1 mice [74]. In another multi-generation study CD (SD) IGS rats were exposed to BPA in the F0 generation beginning at the onset of a premating period and continuing through mating, gestation, and lactation, for two generations. A significant decrease in the frequency of normal estrus cycles in F1 offspring was observed in rats exposed to BPA at 20 μg/kg/day. Some of these females displayed an extended diestrus vaginal smear [76]. In F2 females, no significant changes in the incidence of normal estrus cycles or the length of the estrus cycle were found. BPA had no effect on cycle length or number of days in estrus in 4–8 week old rats [62], 7–11 week old rats [78], 4–5 month old rats [83], 10–13 week old or 11–12 month old rats [88] exposed perinatally to a range of BPA doses. Other studies reported changes in estrus cyclicity after BPA exposure. Sprague-Dawley rats exposed postnatally to BPA at ~ 44 mg/kg/day showed irregular cycles, with a high prevalence of estrus after P90, whereas the length of proestrus and diestrus were markedly reduced [81]. Exposure of Sprague-Dawley rats to BPA from embryonic day 6 until postnatal day 90 at 300 mg/kg/day resulted in an abnormal extension of estrus, when assessed on P69-P90 or on P150-P170. Extension of estrus was also seen on P150-P170 when the exposure dose was 100 mg/kg/day [79]. Asynchrony, defined as a difference of at least two stages of the estrous cycle in either the uterus or vagina compared with the ovary, was prevalent in the 300 mg BPA/kg BW/day group. Estrus cycle was not affected by lower doses of BPA in this study [79]. Wistar rats exposed perinatally to BPA at 1.2 mg/kg/day were less likely to have regular estrus cycles, as determined by monitoring a consecutive 4 week period starting at 3 months of age [84]. Several continuous days of estrus was commonly seen, but in some cases persistent diestrus was observed. In another study, Sprague-Dawley rats exposed to BPA perinatally at 1.2 mg/kg/day also exhibited irregular estrous cycles [85]. The defect of estrus cyclicity varied in individual females and included extended periods of diestrus, proestrus and/or estrus. Persistent diestrus was also seen in 30% of mice exposed to BPA neonatally at either 10 or 100 mg/kg/day [86]. The length of estrus was increased in ICR/Jcl mice exposed to BPA *in utero* at 0.02 and 0.2 mg/kg/day [82]. Exposed animals had a longer cycle overall. Cycle length was also significantly elongated in CD-1 mice treated with BPA *in utero* [80]. The estrus cycle was 5.2±0.1 days in the control group, 8.0±0.4 days in mice exposed to BPA at 0.5 mg/kg/day BPA, and 8.2±0.3 days in mice exposed to BPA at 10 mg/kg/day. The percentage of time spent in the diestrus phase was significantly longer than in untreated controls in this study. In another *in utero* treatment study FVB mice exposed to BPA at 0.5 μg/kg/day spent less time in proestrus and estrus, but more time in metestrus and diestrus, with 20 μg BPA/kg/day causing shortened estrus compared to controls [87].

3.2.3. Fertility—Changes in overall fertility in response to early BPA exposure could indicate impaired uterine physiology. We reviewed nine studies in which the endpoints of BPA exposure include changes in fertility and fecundity [66,74–76,82,87,89–91]. In one study, Crj: CD (SD) IGS rats were continuously exposed to BPA at doses ranging from 200 ng/kg/day to 200 μg/kg/day and 10 week old F1 offspring was mated [76]. No significant effects on fertility index, gestation index, number of implantations, delivery index, and number of F2 pups delivered were observed. The effect of continuous BPA exposure throughout 3 generations at doses ranging from micrograms to hundreds of milligrams per kg/day was investigated in F1 and F2 offspring of CD-1 female mice [74] and Sprague-Dawley rats [75]. Significant decreases in the numbers of implants, total number of pups, and number of live pups per litter at birth and on PND 4 were seen in F2 rats in the 500 mg/kg/day BPA group. No such changes were found in mice or in rats exposed to lower doses. In another two-generation study, untreated ICR/Jcl male mice were mated with 90 to 120 day ICR/Jcl females that had been exposed to BPA *in utero* at 0.02 or 0.2 mg/kg/day [82]. BPA exposure had no effect on the number of offspring in the F2 generation. Similarly, no changes in fertility and fecundity were observed in Long-Evans rats exposed to BPA at 0.02 and 0.2 mg/kg/day from day E7 to P18 and bred continuously for 4 months after weaning [92]. Sprague-Dawley rats exposed during the first 10 postnatal days to 4.35±1.85 mg/kg/day BPA delivered significantly fewer pups at their first pregnancy than control rats, and rats exposed to BPA at 43.75±18.75 mg/kg/day were sterile [89]. Fertility rates were decreased in a dose-dependent manner in neonatally BPA-exposed females (pregnancy rates of 100%, 90%, and 77% at 0, 0.05 and 20 mg/kg/day BPA doses, respectively), with the number of implantation sites decreasing in females at the highest exposure dose [66]. Following *in utero* exposure of FVB mice to BPA at 0.5 or 50 μg/kg/day, litter size was decreased in relation to exposure when assayed in pregnant mice at 3, 6 and 9 months of age [87]. The cumulative effect of early, very low dose BPA exposure on mouse reproductive function was assessed in forced breeding experiments carried out over a 32-week period starting at 2 months of age [91]. A significant decrease in cumulative numbers of pups per dam was seen in mice exposed to BPA at 25 ng/kg/day or 25 μg/kg/day, and this decrease was associated with a significant decrease in total number of litters over the 32 week period in the 25 μg/kg/day exposure group only. These effects were not seen, however, in an intermediate BPA dose (250 ng/kg/day exposure group) [91].

3.2.4. Other uterus-related endpoints—Endpoints other than uterine weight, cyclicity, animal fertility and fecundity were examined in adult animals exposed to BPA prenatally or neonatally. In one study, no effect on uterine histology was seen in 2.5 month-old rats exposed to BPA perinatally [78]. No differences in uterine histology were found in 4, 8, 12 and 16 week old mice after exposure to 0.5 or 10 mg/kg/day BPA from day E15 to E19 [80]. In another study, mice exposed to BPA either prenatally or neonatally (10 or 100 mg/kg/day) were ovariectomized on day P30, and reproductive organ histology was examined on days P40 and P90 [86]. No histological pathology was seen in any of the treatment groups. Among the mice that were not ovariectomized, the number of normally cycling mice was decreased after BPA exposure, as determined on days P61-P90, however, the decrease was not statistically significant. In one study uterine carcinogenicity was initiated by intrauterine administration of N-ethyl-N′-nitro-N-nitrosoguanidine to 11 week old rats exposed

perinatally to BPA at 0.006 mg/kg/day or 6 mg/kg/day [62]. No significant treatment-related differences were observed in the number of neoplastic and preneoplastic lesions upon termination at 15-months of age. In three multigenerational BPA exposure studies, no treatment- or dose-related gross or microscopic changes were seen in uteri of adult F1 to F3 CD Sprague-Dawley rats, F1 adult CD-1 mice, and F1 and F2 mice on PND21 [74–76], although a description of the uterine histopathological analyses is absent from these publications.

In contrast to the negative results reported in the above studies, 15 other studies reported adult uterine effects following early BPA exposure. The thickness of the total epithelium was significantly reduced at estrus in the uterus of 4 month-old rats exposed to 0.1 and 50 mg/kg/day BPA *in utero*. ERα expression was increased in the group exposed to 50 mg/kg/day, and ERβ expression was decreased in the group exposed to 0.1 mg/kg/day [93]. Adenomatous hyperplasia with cystic endometrial hyperplasia was seen in 25% of mice (vs. 10% of control mice) 3 months after perinatal exposure to 0.1 or 1 mg/kg/day BPA [94]. Moreover, there was a large increase in the incidence of endometriosis-like structures, including both glands and stroma found in adipose tissue surrounding the genital tracts of BPA-exposed mice (30–35% in exposure groups vs. only one case in the control group). In a separate study, 4 month-old rats exposed perinatally to ~1.2 mg/kg/day BPA via the drinking water showed the following: increased thickness of the uterine epithelia and stroma, decreased uterine epithelial apoptosis, and down regulation of ERα in uterine epithelial cells on the day of estrus [84]. Cystic endometrial hyperplasia on P90 was significantly increased in the uterus of rats exposed to BPA continuously, starting from embryonic day 6, at a dose of 8 μg or 300 mg/kg/day, but not at intermediate doses [79]. Although the authors consider altered histopathology in the 8 μg/kg/day group as unlikely to be a biologically significant, treatment-related lesion, based on its inconsistent dose-dependence, this finding may reflect the same non-monotonic dose-response relationships reported for many health effects of BPA [95]. Hens exposed to BPA *in ovo* had reduced uterine tubular glandular density and thickness of the tunica mucosa along with decreased hatching at 21 weeks of age [55].

Neonatal exposure to BPA at 0.05 or 20 mg/kg/day impacted uterine responses to steroid hormonal stimuli in adult (day P80) ovariectomized rats, with decreases in sub-epithelial stromal cell proliferation (BrdU incorporation) at both BPA doses [60,96]. Rats exposed to 0.05 mg/kg/day BPA failed to up-regulate ERα in response to hormonal stimulation. Vascular endothelial growth factor, an important regulator of vascular permeability and angiogenesis during the peri-implantation period, was down regulated in rats exposed to BPA at 0.05 or 20 mg/kg/day [96]. Decreased volume of endometrial lamina propria, increased incorporation of BrdU into the endometrial gland epithelial cell DNA, and increased expression of ERα and progesterone receptor were also seen in the luminal epithelium of the endometrium and sub-epithelial stroma in CD-1 mice exposed perinatally to 25 and 250 ng/kg/day of BPA [97].

In rats perinatally exposed to BPA at 0.5 or 50 μg/kg/day, a significant decrease in proliferative activity of glandular epithelium was observed on P90, and in the group exposed to BPA at 50 μg/kg/day, the percentage of glandular perimeter occupied by α-smooth

muscle actin-positive cells was also decreased significantly [88]. In the same study the incidence of morphological changes in the luminal epithelium (cuboidal epithelium instead of tall columnar, abnormal cells) was greater on P360 in both BPA-exposed groups; and in the 50 μg/kg/day exposure group the incidence of glands with cellular anomalies was also higher [88]. Some rats exposed perinatally to BPA were ovariectomized on P360 and uterine responses to E2 were further analysed on P460. The incidence of morphological abnormalities of glands, glands with cellular abnormalities, and glands with squamous metaplasia were more frequent in the BPA-exposed groups. Further, PR expression, but not ERβ expression, was decreased on day P460 in the uterine subepithelial stroma in both BPA exposure groups, while ERα expression was reduced in the 50 μg/kg/day group [88].

Genes that showed altered adult hormone responsiveness following early BPA exposure (developmentally programmed genes) were identified in myometrium of 16 month-old rats exposed to BPA perinatally at 50 mg/kg/day [57]. For this analysis, rats in proestrus and estrus were combined into a single high-estrogen (proliferative phase) group, and correspondingly, rats in metestrus and diestrus were combined into a low-estrogen (secretory phase) group. The effect of neonatal BPA exposure on adult expression of 18 previously identified estrogen-responsive genes was then determined. Five of the 18 genes showed evidence of developmental programming in response to neonatal BPA exposure. Three of the five genes (*Calbindin D9k, Gdf10, Gria2*) were also developmentally programmed by neonatal exposure to DES or genistein, which rendered the genes hypersensitive to estrogenic stimulation during the proliferative phase of the estrus cycle. In contrast, early BPA exposure programmed the same three genes to become repressed by estrogen. The Eker rat model used in this study carries a Tsc2 tumor suppressor gene defect ($Tsc2^{Ek/+}$) and develops uterine leiomyomas by 16 months of age with a historical tumor incidence of 65%. Neonatal BPA exposure did not significantly increase tumor incidence or multiplicity, and it did not induce dramatic morphologic alterations of the reproductive tract.

Other studies of long term (18 months) effects of prenatal [98] or neonatal [99] BPA exposure in mice using low, environmentally relevant doses of BPA revealed increases in cystic endometrial hyperplasia and squamous metaplasia, as well as other, more severe uterine pathologies, including adenomyosis, leiomyomas, atypical hyperplasia, sarcoma of the uterine cervix, and stromal polyps, which were apparently not seen at earlier ages.

3.3. Evidence of epigenetic programming

In this section we considered all studies that report changes in DNA methylation [56,60,100] and histone modification [57] in uteri of exposed animals. *Hox* genes are subject to epigenetic regulation [101,102]; therefore we also considered studies that report changes in *Hox* gene expression [54]. Two of those studies [65,66] are complementary to DNA methylation studies carried out by the same research groups ([56] and [60], respectively). In one study assessing trans-generational effects of BPA exposure, uterine histopathology and differential *Hoxa10* DNA methylation were reported in adult offspring born to mice exposed to BPA neonatally (i.e., F2 generation mice) [100]. However, this study did not control for the estrus cycle stage when the F2 mouse uterine tissue was collected for analysis. Indeed, the data presented indicate large variance in uterine weights in some of the groups (up to

3.7-fold variation; see Table 1 in [100]), consistent with the tissue being harvested at different estrus cycle stages. This study [100] was therefore excluded from further consideration.

A recent study of rhesus macaques exposed to BPA at 400 μg/kg/day during the third trimester (GD100-165) reported changes on gestation day 165 in fetal uterine expression of several genes critical for reproductive organ development and/or adult function, including *HOXA13*, *WNT4* and *WNT5A* [54]. In a study of uterine Hoxa10 regulation [65], Hoxa10 immunoreactive protein was increased up to ~10-fold at both 2 weeks and 6 weeks of age in mice exposed to BPA *in utero* at 0.5, 1 or 5 mg/kg/day. It was not specified whether the 6 week uterine tissue was collected at a fixed point in the estrus cycle; however, it was noted that a similar increase in Hoxa10 was seen in the BPA-exposed group when assessed 2 weeks after ovariectomy performed at 6 weeks of age, in the absence of exogenous hormonal stimulation. A subsequent study from the same laboratory [56] reported the following effects of *in utero* BPA exposure at 5 mg/kg/day: 1) uterine Hoxa10 RNA was increased modestly (by 25%) in adult mice; 2) there was a marked decrease in the mean number of *Hoxa10* promoter and intronic region methylation sites in uterine tissue of offspring at 2 weeks of age but not at adulthood; 3) uterine RNA levels of DNA-methyltransferases Dnmt1, Dnmt3a, and Dnmt3b were not significantly altered at 2 week of age; and 4) ERα binding to an ER motif in the *Hoxa10* promoter was increased following BPA exposure, as determined by chromatin immunoprecipitation analysis using isolated uterine tissue [56]. The significance of the latter finding is difficult to determine, as neither the age of the mice, nor the stage(s) of the estrus cycle at which the tissue samples were collected was specified. The latter factor is critical, given the major changes in ER signaling that occur during the estrus cycle, as well as the significant effects that the estrus cycle can have on the expression of mouse uterine genes, including *Hoxa10* [103].

In another study, female rats were exposed to BPA at 0.05 or 20 mg/kg/day on days P1, P3, P5 and P7, and uterine expression of *Hoxa10* and *Hoxa11* was assayed on day P8 and in ovariectomized adults (P80) stimulated with progesterone + estrogen [60]. *Hoxa10* and *Hoxa11* were significantly down regulated on day P8 and in the hormone-stimulated adults in the 0.05 mg/kg/day BPA exposure group. At the 20 mg/kg/day BPA dose, both *Hoxa* genes were down regulated at P8, but only *Hoxa10* was down regulated at adulthood. BPA exposure did not affect the methylation status of the *Hoxa10* promoter in adult ovariectomized and hormone-stimulated rats, as determined by methylation-specific PCR [60]. In a follow up study, a clear decrease in uterine *Hoxa10* RNA during the late preimplantation period (day 5 of gestation) was seen in impregnated rats exposed neonatally to BPA at both doses [66]. Further, in the day 5 pregnant rat uteri, sub-epithelial levels of Hoxa10 were suppressed at both BPA doses, and two implantation-related genes downstream of Hoxa10 (*Itgb3*, *Emx2*) showed altered expression in the direction that was expected based on the decrease in Hoxa10 levels, albeit only at the higher BPA dose. Similar results were found in mice exposed neonatally to DES at 0.2 μg/kg/day [66].

The ability of BPA and another estrogenic chemical, genistein, to suppress the activity of EZH2, a histone H3-lysine 27 methyltransferase, via non-genomic ER signaling was recently investigated in Eker rats [57]. Genistein, but not BPA (50 mg/kg/day on days P10 to

P12), activated uterine phosphatidylinositol-3 kinase/AKT signaling leading to phosphorylation of EZH2 protein and down regulation of its H3-lysine 27 methyltransferase activity in uteri harvested 6 hr after the last treatment on day 12.

4. Discussion

Published studies of prenatal and perinatal BPA exposure in animal models were evaluated to determine whether there is clear evidence to support the proposal that BPA exposure during a critical window of uterine development permanently programs uterine tissue for development of reproductive abnormalities or increases in disease susceptibility later in life. We found strong and substantial evidence for long term uterine dysregulation in adult animals exposed to BPA at an early developmental stage, based on a large number of studies using different exposure models and a range of BPA doses. While the ability of BPA to program uterine tissue development is thus well established, the mechanisms that underlie this programming are largely unknown. Further, the molecular events that occur during and shortly after early BPA exposure and presumably lead to changes in tissue programming are poorly understood. In particular, as discussed below, there is very limited knowledge of the long-term epigenetic changes that are anticipated to be induced by early BPA exposure, with the current literature in this area largely limited to effects of BPA on uterine *Hoxa* gene methylation.

4.1. Evidence of early signature

Many studies have reported gene expression changes in uteri of pre-pubertal mice and rats, and in one study rhesus monkey, euthanized soon after termination of BPA exposure, however, the molecular events leading to the changes in expression are unknown. Of three available microarray studies, one used a BPA exposure protocol that covers only the very edge of the window of uterine development in rat [64], one measured short-term gene expression responses in pooled ovarian and uterine tissue in rat [59], and one studied what is likely short-term responses in rhesus macaque uterine tissue [54]. Studies of short-term effects of BPA on estrogen-responsive genes indicate that BPA can act as an ER agonist, with induction of genomic ER signaling and trans-activation of gene expression in the developing rat uterus [57]. Many of the studies reviewed here monitored changes in gene expression shortly after termination of BPA exposure, and consequently, a majority of the responses reported may very well be short term, transient effects unrelated to any longer term changes that impact uterine development. The changes in *Hoxa* gene expression seen in early BPA-exposed mice [56,65] and rats [60,66] are an exception, as those outcomes were evaluated at least 20 days after the last dose of BPA was administered – a period long enough to be considered a long term, perhaps life-long change. The changes in *Hoxa* expression seen in these studies may thus be *bona fide* molecular signatures of early BPA exposure. Importantly, the effects on *Hoxa* expression were consistent between pre-pubertal and adult uteri, supporting the proposal that BPA programs the expression of these genes. However, the direction of the effect of BPA on *Hoxa10* expression was not consistent between studies, which could be due to differences in the model species studied and/or differences in the exposure protocols: long-term up regulation of *Hoxa10* was seen in the prenatal mouse exposure model [56,65], while long-term down regulation of *Hoxa10* was

found in the neonatal rat exposure model [60,66]. The significance of these exposure model-dependent differences in *Hoxa* responses is unclear. Changes in expression of several genes critical for reproductive organ development and/or adult function, including *HOXA13*, *WNT4* and *WNT5A* in rhesus macaques exposed to BPA during the third trimester [54], suggest that BPA could alter transcriptional signals influencing uterine function later in life. Thus, data on the ability of BPA to produce a stable molecular signature in developing uterus is scarce and, where present, is inconsistent.

4.2. Evidence of delayed effects

Many of the 37 studies reviewed here investigated the effects of *in utero* or neonatal BPA exposure on adult uterus. Early BPA exposure was reported to have various effects on uterine weight later in life [68–71], however, none of these studies explicitly controlled for the estrus cycle stage when the uteri were collected. Indeed, no effect on uterine weight or effect of only very high dose [75] was seen in seven other studies where the estrus cycle stage when tissues were collected was properly controlled [62,74–79]. Thus, it is likely that adult uterine weight is not sensitive to early BPA exposure. Of 15 studies investigating changes in uterine cyclicity in response to BPA, six studies did not find any significant effects [62,74,75,78,83,88]. Eight other studies reported increases in the frequency of irregular cycles in rats [76,79,81,84,85] and elongated cycles in mice [80,82,86,87], with estrus or diestrus often found to be prolonged. Of nine studies investigating changes in fertility of female rodents in response to early BPA exposure, five studies reported decreases in fertility and/or fecundity [66,76,87,89,91] and four studies reported no significant effects [74,75,82,92]. Where found, differences in results between studies may be explained by differences in experimental design, dosing protocols, and animal models. While these effects on reproductive function are clearly important, these findings do not constitute firm evidence for programming of uterine tissue by early BPA exposure.

Seven studies reported no effects of early BPA exposure on uterine histology and histopathology at adulthood [62,74–76,78,80,86] in contrast, other studies reported altered uterine histology [55,79,84,88,93,94,97], as well as changes in ER expression [84,93,97], responsiveness to hormonal stimulation [57,60,88], and the accumulation of endometriosis-like lesions [94] following early BPA exposure. Another commonly observed uterine outcome of early BPA exposure is endometrial hyperplasia [79,84,94,97–99]. Other outcomes are not entirely consistent between studies. For example, ERα was up regulated in the uterus of rats exposed to BPA at 50 mg/kg/day and in mice exposed to BPA at 25 or 250 ng/kg/day [93,97], but was down regulated in rats exposed to BPA at 1.2 mg/kg/day [84]. Estrogen + progesterone stimulation failed to increase ERα levels in uteri of adult ovariectomized rats exposed to BPA neonatally at 50 μg/kg/day [60], but estrogen stimulation in adult ovariectomized rats exposed to BPA perinatally at 50 μg/kg/day resulted in decreased ERα expression in the subepithelial stroma [88]. Another example of discrepant results involves changes in the epithelial layer thickness at estrus in 4-month old rats. In one study, adult uterine epithelium was significantly increased in thickness after exposure to BPA *in utero* and continuing throughout lactation at ~1.2 mg/kg/day [84], whereas in another study the thickness of the epithelium was significantly reduced after *in utero* exposure to BPA at 0.1 or 50 mg/kg/day [93]. Although there is inconsistency among

the reviewed studies, the overall body of literature points to the ability of a developmental exposure to BPA to induce long-lasting changes in uterine physiology.

Some of these differences in outcome between studies could be due to differences in the exposure models used, including animal species and strains, timing, routes and doses of exposure and timing of outcome assessment. Alternatively, discrepant results such as those cited above might reflect the non-linear (non-monotonic) dose-response relationships (e.g., biphasic or U-shaped dose-response curves) that characterize many BPA outcomes [95]. These discrepancies notwithstanding, the available evidence, when taken as a whole, clearly supports the conclusion that BPA exposure during a critical window of uterine development induces long-term uterine responses, some of which are associated with adverse reproductive or other health effects. Further, the impact of early BPA exposure on uterine tissue hormonal responsiveness may be age dependent: BPA effects may increase in adulthood compared to the immediate post-pubertal period, reflecting the impact of repeated estrogen stimulation during the course of estrus cycling. Further, in some cases BPA effects might not be manifested until the onset of aging, as suggested by the adverse health effects that early low-dose BPA exposures produce in 18-month old mice [98,99].

4.3. Involvement of epigenetic mechanisms

Epigenetic mechanisms are presumed to be an important contributor to the long-term effects of BPA exposure in a variety of settings, however, current knowledge of such mechanisms is quite limited [31]. In the case of the studies of uterine tissue reviewed here, the evidence for epigenetic events associated with programming by early BPA exposure is largely limited to changes in *Hoxa* gene DNA methylation, which has been associated with (but not linked causally to) changes in *Hoxa* gene expression. As noted above, *HOXA13* was differentially expressed in fetal uterine tissue of rhesus macaques exposed *in utero* to 0.4 mg/kg/day BPA [54]. Further, *Hoxa10* was up regulated in mouse uterus pre-pubertally and at adulthood following *in utero* BPA exposure, but was down regulated in rat uterus in both developmental periods following neonatal BPA exposure. Methylation of the *Hoxa10* promoter was decreased in 2 week old mice, however, in both species *Hoxa10* DNA methylation was unaffected at adulthood [56,60]. Thus, the epigenetic actions seen at 2 weeks of age are transient, and the longer-term dysregulation of *Hoxa10* expression remains unexplained. Even more important, the consequences of the observed dysregulation of *Hoxa10* expression, including any potential effects on uterine tissue development, reproductive function and disease susceptibility have yet to be determined.

DNA methylation in mammals is catalyzed by three DNA methyltransferases, Dnmt1, Dnmt3a, and Dnmt3b [104]. Enzymatic demethylation can also occur, and involves DNA repair mechanisms, mainly base excision-repair pathways that activate methyl cytosine demethylation [105]. The steady-state level of DNA CpG methylation is thus a balance between these two opposing processes, and may potentially undergo relatively rapid turnover [106]. Changes in uterine *Hoxa* CpG methylation following BPA exposure could reflect changes in DNA methyltransferase enzyme expression, or activity, and/or changes in methyl-CpG demethylation activity. DNA methyltransferase RNA levels were unaffected by *in utero* BPA exposure in 2 week mouse uteri [56], suggesting that BPA does not induce a

general dysregulation of DNA methyltransferase activity, although that possibility was not directly investigated. The absence of demonstrated long-term effects of BPA on uterine DNA methyltransferase levels contrasts with the permanent changes in Dnmt3a and Dnmt3b expression seen in BPA-exposed prostate [51]. Thus, the mechanisms that underlie changes in uterine DNA-methylation signatures following BPA exposure remain poorly understood.

The molecular events that underlie the changes in CpG methylation of *Hoxa* genes and other specific genomic loci are undoubtedly complex and likely to be linked to other epigenetic changes, in particular, changes in histone modifications affecting chromatin compaction and gene expression [107]. These histone modifications, in turn, are mediated by recruitment of chromatin modifying enzyme complexes and the lincRNA scaffolds on which at least some of these complexes are thought to assemble [108,109]. Most likely, the persistent changes in expression (i.e., programming) of *Hoxa* and other genes in response to early BPA exposure involve changes in the balance between activating and repressive histone marks, in addition to associated changes in DNA methylation. One mechanism by which xenoestrogens may affect global levels of histone marks involves rapid, membrane-activated ER signaling via phosphatidylinositol 3-kinase (PI3K) [110]. Activation of this pathway results in phosphorylation of EZH2 on serine-21, which suppresses EZH2 catalytic activity and leads to a global decrease in histone H3-lysine 27 trimethylation, a repressive chromatin mark [111]. DES and genistein, but not BPA, can activate this non-genomic ER signaling pathway in chromatin of the developing rat uterus [57,110]. In contrast, and consistent with induction of non-genomic phosphatidylinositol-3 kinase/AKT signaling, BPA rapidly (within 30 min) suppresses histone H3-lysine 27 trimethyl levels in the prostate [57]. BPA and other xenoestrogens may dysregulate H3-lysine 27 trimethylation by other mechanisms, as indicated by the up regulation of EZH2 RNA, protein, and activity (global H3-lysine 27 trimethyl levels) in MCF-7 breast cancer cells treated with DES or BPA, and in mammary tissue of mice exposed to BPA *in utero* [40].

4.4. Dose-response data

The use of different endpoints, dosing protocols and animal models in the 37 studies reviewed here complicates the analysis of BPA dose-response relationships, which can be complex [95]. Nine papers do not report any uterine effects of developmental BPA exposure [62,74,75,77,78,83,86,92,112]. These studies cover broad range of BPA doses (0.001–600 mg/kg body weight) and primarily analyze BPA effects on uterine weight, cyclicity, and fertility. Some studies also report histopathology data [62,62,74,75,86]. BPA-induced molecular changes in the uterus were seen at doses ranging from 25 ng/kg [97] to 600 mg/kg [58], with most of the studies reporting molecular changes at BPA doses ranging from tens of micrograms to few milligrams per kg body weight [54,56,59,60,65,66,84,88,93,96]. One outcome that may be attributed to high dose exposure is the change CaBP-9K expression, which was seen only in rats exposed to BPA at 600 mg/kg/day [58,63]. All other responses were reported for a broad range of BPA doses. Taken together, the animal studies reviewed here suggest that developmental exposures to BPA at doses similar to the current US EPA reference dose of 50 μg/kg/day may not be sufficiently low to protect uterine tissue from long-term molecular programming. This is of concern, as the National Toxicology Program Expert Panel has estimated developmental exposure to BPA in the general population

reaches tens of micrograms per kg body weight per day [113]. Other estimations indicate exposure reaches at most a few micrograms per kg body weight per day [114,115]. Current BPA exposure estimations should be used with caution as reasonable estimates of daily intake values based on urinary data are often not possible because of knowledge gaps in BPA toxicokinetics [116], and the ubiquitous use of BPA, which makes it difficult to consider all possible sources of exposure. Recent analysis of more than 80 human biomonitoring studies shows almost ubiquitous exposure of the general population to BPA [8,117], further raising concern about the impact of BPA exposure on human female reproductive health. Our data support the recent decision of EFSA's experts to reduce the safety level for BPA from 50 μg/kg/day to 4 μg/kg/day.

4.5. Knowledge gaps and future directions

This review has identified several major gaps in our knowledge that need to be filled to better understand the long-term effects of BPA and the mechanisms by which early developmental exposure to BPA programs the uterus, as well as other tissues, and the impact of this programming on susceptibility to reproductive dysfunction and adult-onset disease.

First, in spite of a large number of published papers, the variation of experimental parameters and conditions between studies, including animal species and strains, exposure window, dose, route, and timing and outcomes examined, complicates efforts to establish clear dose-response relationships and time-course changes in molecular perturbations based on the data available. Similar difficulties were encountered in the analysis of metabolic programing by BPA [118]. These issues are best addressed through experiments with harmonized design that include an environmentally relevant exposure paradigm and encompass multiple time-points, and that examine outcomes such as those described in the following paragraphs.

Second, the molecular events that BPA activates during and soon after exposure need to be identified on a global scale and elucidated at a mechanistic level. Of particular interest are 1) short-term responses, measured within hours of the last administration of BPA, and 2) early molecular signatures of the exposure, which are best characterized several days after the last exposure but prior to the onset of puberty. Given how little we know about the molecular perturbations that BPA triggers, high-throughput approaches to screening the entire transcriptome (RNA-Seq) and epigenome (e.g., ChIP-Seq analysis of panels of activating and repressive chromatin marks) are essential for characterization of the overall response, and are therefore much preferred over methods and technologies focused on individual genes and specific genomic loci. A time course analysis of BPA-induced estrogen receptor binding to its DNA binding sites in uterine chromatin, recently characterized on a global scale in the mouse model [119], may help elucidate the molecular events that induce uterine tissue programming following a developmental exposure to BPA. Estrogen and estrogen receptors can alter the epigenome by several mechanisms [120]. Only one of these mechanisms, involving induction of non-genomic PI3K/AKT signaling, has been investigated for BPA [57]. Other mechanisms described for estrogen receptor action that may be activated by BPA exposure include recruitment of histone acetyl transferases p300 and CBP and co-activators of the p160 family [121], interaction with MLL1 and other SET1

family lysine methyltransferases, and with KDM4/JMJD2 proteins, which lead to chromatin activation by respectively catalyzing methylation of histone-H3 lysine 4 and demethylation of histone-H3 methylysine 9 [122,123], and recruitment of CARM-1, which regulates estrogen receptor-mediated transcription via arginine methylation of receptor-associated coactivator proteins [124].

Third, further work is needed to characterize the interactions of BPA-dysregulated molecular pathways with estrogens and other endogenous hormones throughout life, and to elucidate whether, and how, these interactions facilitate the emergence and/or amplification of the initial phenotypic abnormalities induced by early xenoestrogenic exposure. Such studies could employ high throughput analysis of the interactions of molecular pathways dysregulated by early BPA exposure in the context of physiological steroid stimulation given to ovariectomized adult animals. Comparisons of outcomes between adult animals ovariectomized at the beginning of puberty versus animals ovariectomized as young adults, i.e., after experiencing several natural estrus cycles, may help identify molecular pathways and epigenetic events whose dysregulation by early BPA exposure is triggered by repeated cycles of estrogen stimulation.

Fourth, given the extensive, cyclic remodeling that uterine tissue undergoes throughout the estrus/menstrual cycle, with 1,000 or more genes showing altered expression during the course of the cycle [103,125], it is important to determine which of the pathways orchestrating normal tissue remodeling may be permanently altered by early developmental exposure to BPA. It is also important to ascertain the consequences of these alterations for the cycling uterus. Surprisingly, 7 of the 44 peer-reviewed publications initially considered in this study failed to explicitly control for the stage of the estrus cycle when tissues were excised and analyzed for outcomes that are known to be dependent on estrus cycle stage, e.g., uterus weight and expression of key developmental genes. Clearly, biological effects, gene expression changes and epigenetic outcomes identified in such studies may reflect differences in estrus cycle stage between control and treatment groups, and cannot be reliably attributed to BPA exposure.

5. Conclusions

Our analysis of 37 available uterine-related publications where laboratory animals were exposed to BPA during the period of uterine tissue organogenesis and differentiation, and with proper control of estrus cycling, leads us to the following conclusions regarding the impact of an early developmental exposure to BPA on uterine tissue programming and predisposition to adult-onset disease: 1) there is clear evidence for effects of early, developmental exposure to BPA leading to uterine abnormalities during adulthood in mice and rats; 2) there is a poor understanding of the mechanisms that contribute to uterine tissue programming by BPA, although perturbation of one or more epigenetic regulatory mechanisms is highly likely; and 3) high quality genome-wide data sets are needed to characterize on a global scale changes in gene expression and epigenetic remodeling and to elucidate the mechanisms of perturbation of molecular pathways in uterine tissue. Careful examination of three key time periods is needed to fully elucidate both the initial response to the exposure and its long term consequences: an initial, short-term response time point; a

delayed, post-exposure but pre-pubertal time point, to identify molecular signatures of the exposure; and one or more longer-term time points, to assess life-long responses that are manifested at adulthood and in aged animals.

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Abbreviations

BPA	bisphenol A
ER	estrogen receptor

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Highlights

- Developmental uterine effects of Bisphenol A were analyzed in 44 experimental studies.
- Developmental exposure to BPA leads to uterine abnormalities in adulthood in mice and rats.
- Mechanisms of uterine programing are unknown although epigenetic mechanisms are likely involved.
- Genome-wide data sets are needed to characterize changes in gene expression and epigenetic remodeling by BPA.
- Examination of three time periods is needed: short-term response, delayed pre-pubertal time point, and longer-term adult time point(s).

Table 1

Experimental design and outcomes of the 37 studies selected for systematic review, after filtering to remove 7 studies in which estrus stage-dependent outcomes were analyzed without proper control of estrus cycle (see text). Studies are listed in the order presented in the text

Model	Exposure dose (mg/kg/day)	Lowest effective dose (mg/kg/day)*	Exposure window	Route**	OT***	Outcome assessment (age)	Outcome type	Reference
Rhesus macaque	0.4	0.4	E50-100	po to dams	--	E100	Histology, ERα, PR, Ki-67 immunohistochemistry, all genome gene expression	[54]
		0.4	E100-165			E165		
White Leghorn hens	67, 134	134	E4	in ovo	--	P147	Histopathology, ERα expression	[55]
CD-1 mice	5	5	E9-E16	ip to dams	--	P14, P42	Expression and methylation of *Hoxa10*	[56]
Eker rats	50	50	P10-P12	sc	--	P12	Expression of 18 ERE genes	[57]
		50	P10-P12	sc	--	P480	Expression of 18 ERE genes	
		--	P10-P12	sc	--	P90, P480	Tumor incidence, non-genomic ER signaling, activity of EZH2, histopathology	
Eker rats	0.00005, 0.05, 50	--	P12	sc	--	P12	non-genomic ER signaling	
Sprague-Dawley rats	0.00005, 0.01 0.05, 50	--	P12	sc	--	P12	non-genomic ER signaling	
Sprague-Dawley rats	0.0004–0.05	--	P12	po	--	P12	non-genomic ER signaling	
Sprague-Dawley rats	200, 400, 600	600	P1-P5	sc to dams	--	P6	CaBP-9k expression	[58]
Sprague-Dawley rats	5, 50, 400	5	E11-E20	sc to dams	--	E20	Transcriptome of uterus pooled with ovaries	[59]
Wistar-derived rats	0.05, 20	0.05	P1, P3, P5, P7	sc	--	P8	*Hoxa10*, *Hoxa11* expression	[60]
	0.05, 20	0.05	P1, P3, P5, P7	sc	P80	P93 (after hormonal stimulation)	ERα, PR, Hoxa10, Hoxa11, SMRT, SPC-3 expression, DNA synthesis, *Hoxa10* promoter methylation	
Donryu rats	0.006, 6	--	E2-P21	po to dams	--	P14, 21, 28, 56	Uterus weight	[62]
		--				P10, 14, 21, 28	Uterus gland development, ERα immunohistochemistry	
		--				~ P30-56	Cyclicity	
		--				P77-450	Carcinogenicity after N-ethyl-N′-nitro-N-nitrosoguanidine intrauterine administration	

Model	Exposure dose (mg/kg/day)	Lowest effective dose (mg/kg/day)*	Exposure window	Route**	OT***	Outcome assessment (age)	Outcome type	Reference
Sprague-Dawley rats	200, 400, 600	600	E17-19	sc to dams	--	P5	CaBP-9k expression	[63]
Sprague-Dawley rats	600	600	P14-P16	sc to dams	--	P17	Transcriptome	[64]
CD-1 mice	0.5, 1, 5, 50, 200	0.5	E9-E16	ip to dams	--	P14, P42	Hoxa10 immunohistochemistry	[65]
Wistar-derived rats	0.05, 20	0.05	P1, P3, P5, P7	sc	--	> P80 at pregnancy	Reproductive performance, PR, ERα immunohistochemistry, and gene expression	[66]
Crj: CD (SD) IGS rats	0.0002, 0.002, 0.02, 0.2	0.02	E1-∞	po	--	>P70	Cyclicity of F1 and F2	[76]
		--					Fertility of F1, uterus weight, histology F1-F2	
CD-1 mice	~ 0.003, 0.03, 0.3, 5, 50, 600	--	E1-∞	po	--	~ P56-112 (F1)	Cyclicity (for 3 weeks), fertility	[74]
		--				P21 (F1, F2)	Uterus weight, histopathology	
Sprague-Dawley rats	~ 0.001, 0.02, 0.3, 5, 50, 500	500	E1-∞	po	--	~ P70-117	Fertility (F2)	[75]
		--				~ P70-117	Cyclicity (for 3 weeks, F1-F3), Fertility (F1)	
		--				P21 (F1, F2)	Uterus weight	
		500				F1-2 dams after weaning	Uterus weight	
		--				F1-3 dams	Histopathology	
Sprague-Dawley, Alderley Park (Wistar derived) rats	0.02, 0.1, 50	--	E6-E21	po to dams	--	P98	Uterus weight	[77]
		50					Cycle onset	
Sprague-Dawley rats	231.8 at gestation, and 384.4 at lactation	--	E15-P10	po to dams	--	P77 at diestrus	Cyclicity (P48-P77), histology, uterus weight	[78]
Sprague-Dawley rats	0.0025, 0.008, 0.025, 0.08, 0.26, 0.84, 2.7, 100, 300	100	E6-P90	po	--	VO****+10 days, P69-90, P150-170	Cyclicity	[79]
		n/a				P21, P90 at estrus	Uterus weight	
		0.008				P90	Histopathology	
CD-1 mice	0.5, 10	0.5	E15-19	sc to dams	--	P63-77	Cyclicity	[80]
		--				P28, 56, 84, 112	Histopathology	
Sprague-Dawley rats	4.35±1.85, 43.75±18.75	43.75±18.75	P1-P10	sc	--	P60-120	Cyclicity	[81]
ICR/Jcl mice	0.02, 0.2	0.02	E11-17	sc to dams	--	P26-56	Cyclicity	[82]
		--				P90-120	Fertility (pups number)	

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Model	Exposure dose (mg/kg/day)	Lowest effective dose (mg/kg/day)*	Exposure window	Route**	OT***	Outcome assessment (age)	Outcome type	Reference
Crl:CD BR Sprague-Dawley rats	3.2, 32, or 320	--	E11-P20	po	--	P120-142	Cyclicity	[83]
Wistar rats	1.2	1.2	E6-P21	po to dams	--	P120, at estrus	Cyclicity, ERα immunohistochemistry, histopathology	[84]
Sprague-Dawley rats	~0.1, 1.2	--	E6-P21	po to dams	--	P1, 240, 360–480	Macroscopic abnormalities	[85]
		1.2				P120-138	Cyclicity	
ICR/Jcl mice	10, 100	--	E10-E18	sc to dams	P30	P40	Histopathology	[86]
	~ 10, 100	~ 100	P1-P5	sc	P30	P40, P90	Histopathology	
		~ 10			--	P61-P90	Cyclicity	
		--				F1 dams after weaning (~13 week)	Uterus weight, histopathology	
FVB mice	0.0005, 0.02, 0.05	0.0005	E11-E21	po to dams	--	VO***+30 days	Cyclicity	[87]
		0.0005				3, 6, 9 months	Fertility	
Wistar-derived rats	0.0005, 0.05	0.0005	E9-P21	po to dams	--	P90, 360 in estrus	Histology, α SMA, vimentin, immunohistochemistry, proliferative, apoptotic activity	[88]
		n/a				P70-90, 340-360	Cycling	
		0.0005			P360	P460	Histology, ERα, ERβ and PR immunohistochemistry, expression of IGF1, IGFR, p63	
Sprague-Dawley rats	0.44±0.19, 4.35±1.85, 43.75±18.75	4.35±1.85	P1-P10	sc	--	P120-150	Fertility	[89]
CD-1 mice	0.000025, 0.00025, 0.025	0.000025	E8-P16	pump to dams	--	P60	Number of pups and pregnancies	[91]
Long-Evans rats	0.02, 0.2	--	E7-P18	po to dams	--	P23-143	Fertility and fecundity	[92]
Sprague-Dawley rats	0.1, 50	0.1	E6-E21	po to dams	--	P120 at estrus	Histopathology, ERα and ERβ immunohistochemistry	[93]
Balb-C mice	0.1, 1	0.1	E1-P7	sc to dams	--	P90	Histopathology, ectopic endometrium	[94]
Wistar rats	0.05, 20	0.05	P1, P3, P5, P7	sc	P80	P93	Vegf expression, endothelial proliferation	[96]
CD-1 mice	0.000025, 0.00025	0.000025	E9-P4	pump to dams	--	P90, at proestrus	ERα, PR expression, histopathology, DNA synthesis	[97]
CD-1 mice	0.0001, 0.001, 0.01, 0.1, 1	0.001	E9-E16	sc to dams	--	P540	Histopathology	[99]

Model	Exposure dose (mg/kg/day)	Lowest effective dose (mg/kg/day)*	Exposure window	Route**	OT***	Outcome assessment (age)	Outcome type	Reference
Wistar rats	0.025, 0.25, 5, 50	--	E7-P22	po to dams		P16	Uterus weight	[112]
CD-1 mice	0.01, 0.1, 1	0.01	P1-P5	sc	--	P540	Histopathology	[126]

* (n/c) – no conclusive results

** ip – intraperitoneal injection, sc – subcutaneous injection, po – per oral administration, pump – osmotic pumps implanted subcutaneous

*** OT - Ovariectomy

**** VO – day of vaginal opening