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NTP Research Report on the Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity (CLARITY-BPA): A Compendium of Published Findings

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Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

NTP reports the findings from many of its studies in the NTP Technical Report and Monograph series. NTP uses the Research Report series, which began in 2016, to report on work that does not fit readily into one of those two series, such as pilot studies, assay development or optimization studies, literature surveys or scoping reviews, and handbooks on NTP procedures or study specifications.

NTP Research Reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these evaluations are included in NTP's Chemical Effects in Biological Systems database or the Health Assessment and Workspace Collaborative.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Peer Review

The draft *NTP Research Report on the Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity (CLARITY-BPA): A Compendium of Published Findings* (formerly titled the *Draft NTP Research Report # 23: CLARITY-BPA Final Report*) was evaluated by the reviewers listed below. These reviewers served as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determined if the report fully and clearly presents a summary of the methods, reported findings, and conclusions of the publications of the CLARITY-BPA study.

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Disclaimer

This report should not be construed to represent FDA's views or policies. This report provides a partial account of the findings and conclusions of the CLARITY-BPA core study conducted by FDA/NCTR staff and of the investigational studies conducted by each individual consortium grantee, as reported in their respective peer-reviewed publications. The summary of each peer-reviewed publication is the sole responsibility of the respective study authors, and participation in this report does not imply acceptance or agreement on the data reporting or interpretation in each peer-reviewed publication.

Abstract

The Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity (CLARITY-BPA) is a multi-agency research program developed by the National Toxicology Program. It was designed to draw upon the strengths of regulatory expertise and research approaches and academic expertise to fill knowledge gaps, enhance quality control, inform chemical risk assessment, and identify new methods or endpoints for regulatory hazard assessments. Bisphenol A (BPA) was chosen as the test chemical because of its widespread low-level human exposure. Participants in the program included researchers from the U.S. Food and Drug Administration who conducted the core guideline-compliant studies and 14 university-based researchers who were supported by grants from the National Institute of Environmental Health Sciences. Using a Sprague Dawley rat model, animals were orally dosed with BPA (2.5, 25, 250, 2,500, or 25,000 μg/kg body weight [bw]/day) beginning on gestation day (GD) 6 and continuing through postnatal day (PND) 21 (stop dose) or 2 years (continuous dose). As a positive estrogen control, animals were similarly dosed with ethinyl estradiol ([EE2]; 0.05 or 0.5 μg/kg bw/day) from GD 6 through 2 years of age. This report presents a collation of the reported findings and the peer-reviewed conclusions from the CLARITY-BPA core guideline study (NTP 2018) and the 19 peer-reviewed publications of the investigational research arm. The report is organized into 10 chapters by organ or organ system, including brain and behavior, cardiac, immune, mammary gland, ovary, penile function, prostate gland and urethra, testis and epididymis, metabolism and thyroid hormone, and uterus. This report provides a succinct summary of the experimental procedures, findings, and authors' interpretations reported in each of the peer-reviewed publications. This report does not attempt to integrate the findings or offer interpretation of reported findings.

Key words: bisphenol A, endocrine disrupting chemical, endocrine active chemical, ethinyl estradiol, guideline toxicology studies, mechanistic studies

Executive Summary

The National Toxicology Program (NTP), National Institute of Environmental Health Sciences (NIEHS), Food and Drug Administration (FDA), and a group of NIEHS grant-supported researchers developed a consortium-based research program to link investigational studies with a modified guideline-compliant toxicology study using a rat model. Bisphenol A (BPA), a chemical with widespread low-level human exposure, was selected as the test chemical because of "the diverse and often difficult-to-interpret evidence" on its health effects (Schug et al. 2013). The resulting research program was called the Consortium Linking Academic and Regulatory Insights on Bisphenol A (BPA) Toxicity (CLARITY-BPA) (Schug et al. 2013). Sprague Dawley rats from the FDA's National Center for Toxicological Research (NCTR) colony were housed, bred, and orally dosed with BPA in a 2-year perinatal dosing study (called the core study) that was performed at NCTR. Siblings of the rat offspring generated in the core study were provided to the grantees at 14 university laboratories for investigational studies that assessed other endpoints, many of which are not typically evaluated in guideline toxicological studies.

In brief, the core study assessed exposures to a wide range of BPA doses (2.5–25,000 μg/kg body weight [bw]/day) beginning in utero on gestation day (GD) 6 and continuing through postnatal day (PND) 21 (stop-dose arm) or for 2 years (continuous-dose arm). Similar continuous exposure to two levels of ethinyl estradiol (variously referred to as EE or EE2; administered at 0.05 and 0.5 μg/kg bw/day) served as a reference estrogen control, and continuous- or stop-dose exposures to the gavage vehicle served as negative controls. Dosing was done daily by oral gavage and included direct dosing of the pups from birth through weaning to ensure exposure during the perinatal period and to overcome the reported poor lactational transfer of BPA. Assessments in the core study included in-life body weight and clinical observations, estrous cyclicity, sperm parameters, organ weights, and clinical pathology at 1 year and complete necropsy and histopathology at 1 and 2 years. Assessments in the grantee studies focused on earlier timepoints than did the guideline core study and included functional, morphological, and molecular endpoints in the animals as early as PND 1 through age 1 year. The study design of the CLARITY-BPA program was structured to reduce many potential sources of bias. These study design features included such things as randomized allocation of animals to the study, use of a single pup per sex per litter for a given endpoint to control for litter effects, monitoring the purity of BPA and accuracy of dosing, quantification of the background dietary intake of BPA, and blinding of the treatment group of the biological samples, among others. To provide context, the BPA dose range included doses above and below the current United States Environmental Protection Agency reference dose of 50 μg/kg bw/day (U.S. EPA 1988).

This report presents a collation of the reported findings and the peer-reviewed conclusions from the CLARITY-BPA core guideline study (NTP 2018) and the peer-reviewed publications of the investigational research arm, which are listed in Table 1. The findings from the core study presented in the following chapters include all measurements found significant ($p < 0.05$) by the statistical tests applied, which in the case of histopathology data did not include adjustment for multiple comparisons. The report is organized into 10 chapters by organ or organ system, including brain and behavior, cardiac, immune, mammary gland, ovary, penile function, prostate gland and urethra, testis and epididymis, metabolism and thyroid hormone, and uterus. This report provides a succinct summary of the experimental procedures, findings, and authors'

interpretations reported in each of the peer-reviewed publications. The reader may refer to the raw data in the NTP's Chemical Effects in Biological Systems (CEBS) database and study details in the original peer-reviewed publications for complete details. In addition, Camacho et al. (2019) presented a summary of the core study data but included a more expansive discussion of the FDA's conclusions. An integrated analysis of findings from eight CLARITY-BPA grantee studies has also been published (Heindel et al. 2020); however, this paper is not summarized in this report.

Several grantee studies remain unpublished, and these studies are noted in Table 1. The designs of these studies are briefly described in the following chapters, but their findings are not included. All data from the core study and the majority of data from the grantee studies, including unpublished studies, are available in the NTP Chemical Effects in Biological Systems (CEBS) database: https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA . The data for the remaining two studies of brain and behavior that evaluated tissues from the CLARITY-BPA program, but were not funded by the program, are available upon request from Dr. Heather Patisaul (Arambula et al. 2018; Witchey et al. 2019).

Summary of Authors' Conclusions from CLARITY-BPA Peer-reviewed Publications to Date

The following section of the Executive Summary briefly states the conclusions of each author from their own publication(s) for each organ or organ system. The NCTR authors' overall conclusions from the core study are provided because the authors did not present conclusions for each organ or organ system separately. Findings from the core study and each individual investigational study are provided in the body of this report in the respective organ or organ system chapter.

Chapters 1 through 10, All Outcomes, Core Study

Given the peer-reviewed findings of the core study, the authors concluded that, "There were few significant effects of BPA treatment in the in-life data collected. […] For clinical pathology endpoints and organ weights, some statistically significant effects of continuous- or stop-dose BPA treatments were observed. These effects were of questionable relevance to BPA toxicity given that they were seen only in single-dose groups, in several cases differed from the vehicle control by less than 10%, and, in the case of organ weights, were not significant when adjusted for body weight" (NTP 2018). In the histopathological evaluations, "statistical differences between BPA treatment groups, particularly below 25,000 µg/kg bw/day, and the vehicle control group detected by the low-stringency statistical tests applied to histopathology lesions, were not dose responsive, sometimes occurring in only one low or intermediate dose group and did not demonstrate a clear pattern of consistent responses within or across organs within the stop- and continuous-dose arms and sacrifice times. In contrast, the high EE2 dose elicited several estrogenic effects in females in a clearly interpretable and biologically plausible manner. Several observations at 25,000 µg BPA/kg bw/day may be treatment related, including effects … in the female reproductive tract (ovary, uterus, and vagina) and in the male pituitary" (NTP 2018). The authors determined, "Based on a weight of evidence approach, we conclude that the core study data do not suggest a plausible hazard of BPA exposure in the lower end of the dose range tested" (Camacho et al. 2019).

Chapter 1, Brain and Behavior, Grantee Studies

The Patisaul laboratory reported from its studies that exposure to BPA did not affect anxiety or exploratory behavior in juveniles or adults but did result in changes in the expression of genes (including estrogen receptors, androgen receptors, and other genes involved in sexual differentiation) and neuroendocrine function in the hypothalamus, hippocampus, and amygdala as early as PND 1. Structural changes in the hypothalamus and amygdala, as well as loss of sex differences in hypothalamic oxytocin receptor levels, were reported on PND 28. Changes were observed across the entire exposure range. The authors concluded that "these data demonstrate prenatal BPA exposure, even at doses below the current no-observed-adverse-effect level, can alter gene expression in the developing brain" (Arambula et al. 2016).

In its studies, the Rosenfeld laboratory also found a loss of sexual dimorphism in expression levels and promoter DNA methylation of selected genes in the hippocampus and hypothalamus and observed some evidence of disruption of spatial navigational learning and memory at the 2,500 μg BPA/kg bw/day exposure level in females. The authors concluded that "findings suggest BPA exposure induced non-EE-like gene expression and epigenetic changes in adult rat hippocampi, a region involved in spatial navigation" (Cheong et al. 2018).

Chapter 2, Cardiac, Grantee Study

The Belcher laboratory reported from its studies that in BPA- or EE2-exposed females, "cardiomyopathy incidence and severity was significantly increased compared to control females at PND 21 with myocardial degeneration observed in both males and females at PND 21 and PND 90" (Gear et al. 2017).

Chapter 3, Immune, Grantee Studies

The Kaminski laboratory evaluated leukocyte composition or function at PND 21, PND 90, 6 months, and 1 year of age. In total, of the 1,160 measurements in BPA-treated rats, 45 measurements were statistically different from vehicle controls. Changes were associated with the percentage of macrophage or dendritic cell populations in the spleen primarily in 6-month-old or 1-year-old males. In addition, changes in T cell activation in 6-month-old males and lymphoproliferative response in 1-year-old males were observed at doses from 2.5 to 25,000 μg BPA/kg bw/day. With the exception of the aforementioned changes, the authors noted that those associated with BPA treatment were mostly sporadic, moderate in magnitude, not dose-dependent, and showed no persistent trend over the 1-year time period examined. Given these findings, the authors concluded that the "BPA-mediated changes observed in the study are unlikely to alter immune competence in adult rats" (Li et al. 2018a).

Chapter 4, Mammary Gland, Grantee Study

The Soto laboratory reported that, using both semiquantitative and newly developed quantitative methods of analyzing mammary gland development, BPA exposure showed nonmonotonic effects. The new quantitative methods and software were specifically designed to assess and quantify multiple endpoints (including 91 structural mammary gland endpoints) at PND 21, PND 90, and 6 months of age. Using permutation tests and additional statistical tools examining a variety of dose-response relationships, they reported that three-dimensional (3D) morphometric measurements of mammary gland whole mounts showed nonmonotonic effects of BPA with a breaking point between the 25 and 250 μg/kg bw/day doses. Most of the set of 91 endpoints showed the same breaking point, and the statistical analysis showed that this was not a random occurrence. Further, for many of the developmental and histoarchitectural endpoints examined, BPA and EE2 had different effects. The authors' concluded that "At all time points, lower doses

resulted in larger effects [than higher doses of BPA], consistent with the core study, which revealed a significant increase of mammary adenocarcinoma incidence in the stop-dose animals at the lowest BPA dose tested" (Montévil et al. 2020).

Chapter 5, Ovary, Grantee Study

The Flaws laboratory concluded from its studies that exposure to lower doses of BPA (2.5 and 250 µg/kg bw/day) decreased the numbers of primordial, primary, preantral, and total healthy follicles at PND 21. At the highest doses tested, BPA exposures reduced circulating estradiol levels in continuous-dose females at 1 year. The authors concluded that "BPA exposures at some doses and time points affect ovarian follicle numbers and sex steroid levels in the rat, but these effects are different than those observed with ethinyl estradiol exposure and some previous studies on BPA" (Patel et al. 2017).

Chapter 6, Penile Function, Grantee Study

Findings from the Gonzalez-Cadavid laboratory have not been published in a peer-reviewed publication. The study data are available in the CEBS database (https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA).

Chapter 7, Prostate Gland and Urethra, Grantee Studies

The Prins laboratory reported that "…although developmental and chronic BPA exposures are not carcinogenic to the prostate, they enhance carcinogenic susceptibility [of the lateral prostate and dorsolateral prostatic ducts] to later-life estrogen exposures, with the greatest effects observed at the lowest BPA dose examined" (Prins et al. 2018). Furthermore, chronic low-dose BPA exposures altered adult dorsolateral prostate stem cell homeostasis with increases in stem cell numbers (2.5 µg BPA/kg bw/day) and progenitor cell proliferation (25 and 250 µg BPA/kg bw/day) and a shift in lineage commitment toward basal progenitor cells (25 and 250 µg BPA/kg bw/day), "which may underpin the increased carcinogenic risk with aging" (Prins et al. 2018).

The vom Saal and Ricke laboratories reported that their morphometric analysis of 3D reconstructions of the rat urogenital sinus (UGS; prostate and urethra) on PND 1 "revealed several significant or trending changes […], including changes in colliculus angle and an increase in colliculus size, a decrease in urethral lumen length and width due to low, but not high doses of BPA, similar to EE2. These changes all indicate that the urethra is smaller, associated with changes in colliculus shape, when exposed to low doses of BPA and EE2" (Uchtmann et al. 2020). The authors concluded that "…these data suggest that BPA at lower doses (physiologically relevant [2.5, 25, and 250 µg/kg bw/day]), but not the highest dose, mediates its effects on the fetal UGS via estrogenic pathways" and "…provide further evidence that BPA mediates nonmonotonic developmental effects on the fetal urogenital sinus" (Uchtmann et al. 2020).

Chapter 8, Testis and Epididymis, Grantee Study

The Boekelheide laboratory reported in its studies that perinatal exposure to 250,000 µg BPA/kg bw/day lowered testis and epididymal weights, but that "prolonged exposure starting in utero to BPA over a wide range of levels [2.5–25,000 µg BPA/kg bw/day] has little, if any, impact on the testes and sperm molecular profiles of 90 day old rats as assessed by the histopathologic, morphometric, and molecular endpoints evaluated" (Dere et al. 2018).

Chapter 9, Metabolism and Thyroid, Grantee Studies

Findings from the Ben-Jonathan and Greenberg laboratories have not been published in a peer-reviewed publication. The study data are available in the CEBS database (https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA).

The Zoeller laboratory reported in its studies that "neither BPA nor EE affected serum thyroid hormones or thyroid hormone–sensitive end points in the developing brain at PND 15. In contrast, propylthiouracil (PTU) reduced serum T4 to the expected degree (80% reduction) and elevated serum TSH [thyroid stimulating hormone]. Few effects of PTU were observed in the male brain and none in the female brain. As a result, it is difficult to interpret the negative effects of BPA on the thyroid in this rat strain because the thyroid system appears to respond differently from that of other rat strains" (Bansal and Zoeller 2019).

Chapter 10, Uterus, Grantee Study

The Ho laboratory reported minimal, non-statistically significant effects when effects of BPA exposures were evaluated on individual uterine endpoints in isolation. When the combined effects of all testing outcomes were analyzed in a semi-blind fashion using statistical models, however, the authors concluded that "life-long exposure to low doses of BPA (25 or 250 μg/kg/day), but not higher doses, altered the estrous cycle and uterine pathology." Furthermore, over 400 genes were differentially expressed in each BPA-exposed group relative to control, and expression patterns of genes from the 25 and 250 μg/kg bw/day dose groups were most closely related to each other. The 25 and 250 μg/kg bw/day dose groups also had greater gene expression similarity to the high-dose EE2 group than to the other BPA groups or the low-dose EE2 group, which were all more likely to be similar to the control group. A subset of BPA-responsive genes from the 25 and 250 μg BPA/kg bw/day dose groups was also linked to estrogen responsiveness and "exhibited a non-monotonic dose-response pattern" (Leung et al. 2020).

Table 1. CLARITY-BPA Program Studies by Outcome Measured and Timing of Assessment

	PND 1	PND 15	PND 21	PND 25–28	PND 90	PND 97–125	16 ± 2 wk or 20−22 wk	6 mo	1 yr	2 yr
Brain and Behavior	Patisaul (Arambula et al. 2016; Arambula et al. 2018)	–	–	Patisaul (Arambula et al. 2017; Rebuli et al. 2015; Witchey et al. 2019)	–	Patisaul (Rebuli et al. 2015) and Rosenfeld (Cheong et al. 2018; Johnson et al. 2016)	–	–	Core (NTP 2018)[1]	Core (NTP 2018)
Cardiac	–	–	Belcher (Gear et al. 2017)	–	Belcher (Gear et al. 2017)	–	–	Belcher (Gear et al. 2017)[2]	Core (NTP 2018)	Core (NTP 2018)
Immune	–	–	Kaminski (Li et al. 2018b)	–	Kaminski (Li et al. 2018a; 2018b)	–	–	Kaminski (Li et al. 2018a; 2018b)[2]	Kaminski (Li et al. 2018a; 2018b), Core (NTP 2018)	Core (NTP 2018)
Mammary Gland	–	–	Soto (Montévil et al. 2020) Soto (Unpublished)	–	Soto (Montévil et al. 2020)	–	–	Soto (Montévil et al. 2020)[2]	Core (NTP 2018)	Core (NTP 2018)
Ovary	Flaws (Patel et al. 2017)	_	Flaws (Patel et al. 2017)	–	Flaws (Patel et al. 2017)	–	Core (NTP 2018)	Flaws (Patel et al. 2017)[2]	Flaws (Patel et al. 2017), Core (NTP 2018)	Core (NTP 2018)
Penile Function	–	–	–	–	–	–	–	Gonzalez-Cadavid (Unpublished)	–	–
Prostate Gland and Urethra	vom Saal (Uchtmann et al. 2020)	–	–	–	–	–	–	Prins (Prins et al. 2018)[2]	Prins (Prins et al. 2018) Prins (Unpublished), vom Saal (Unpublished), Core (NTP 2018)	Core (NTP 2018)

	PND 1	PND 15	PND 21	PND 25–28	PND 90	PND 97–125	16 ± 2 wk or 20–22 wk	6 mo	1 yr	2 yr
Testis and Epididymis	–	–	–	–	Boekelheide (Dere et al. 2018)	–	–	–	Core (NTP 2018)	Core (NTP 2018)
Metabolism and Thyroid	–	Zoeller (Bansal and Zoeller 2019)	–	–	Ben-Jonathan (Unpublished)	–	–	Ben-Jonathan (Unpublished)[2]	Greenberg (Unpublished), Core (NTP 2018)	Core (NTP 2018)
Uterus	–	–	Ho (Leung et al. 2020)	–	Ho (Leung et al. 2020)	–	–	Ho (Unpublished)[2]	Ho (Leung et al. 2020), Core (NTP 2018)	Core (NTP 2018)

Unpublished raw data are available in the National Toxicology Program's Chemical Effects in Biological Systems or by contacting the author.
mo = month; PND = postnatal day; wk = week; yr = year.
[1]Camacho et al. (2019) presented a summary of the core study data but included a more expansive discussion of the FDA's conclusions.
[2]An integrated analysis of findings from eight grantee studies has also been published (Heindel et al. 2020). The manuscript included data from 6-month-old animals for cardiac, immune, mammary gland, metabolism, ovary, prostate, and uterus; this analysis is not summarized is the CLARITY-BPA Compendium Report.

Introduction

Background

Current methods for assessing the toxicity of chemicals are primarily based on two main sources of information: "investigational" research and guideline-compliant studies. Investigational research is typically conducted by investigators who are usually employed at a university and fund their research through federal or other sourced grants. Investigational research studies tend to be hypothesis-driven studies that are conducted on a relatively modest scale using a variety of experimental models to characterize a chemical's effect on a variety of biological or disease-related endpoints. In contrast, guideline-compliant studies are typically larger-scale efforts that follow established validated protocols for assessing toxicity and, often, to meet regulatory needs. Guideline studies are usually conducted in accordance with good laboratory practices (GLP), which are a set of internationally recognized quality assurance and quality control processes developed to standardize the conduct, record-keeping, and reporting of study results. Because of the extensive documentation requirements, guideline and GLP studies require a high level of quality control and management oversight and, thus, are generally conducted by accredited government or commercial laboratories.

Both investigational research and guideline-compliant studies make important contributions to the understanding of the potential health effects of chemicals, and both types of studies are taken into account at the regulatory level. As stated in Schug et al. (2013), "The strengths of academic research include a greater flexibility to respond to new scientific developments and experiment with new technologies, methods, or less-characterized endpoints. Hypothesis-driven academic research is also often driven to explore fundamental mechanisms of biological phenomena, not just assessment of potential toxicity or risk. On the other hand, guideline studies conducted in accordance with GLP benefit from validated methods and established protocols, transparent data recording and reporting systems, and a level of standardization, quality control, statistical power, and consistency that allows results to be compared more easily among studies."

Bisphenol A

Bisphenol A (BPA) is a chemical used to produce polycarbonate plastics and epoxy resins. It is used in the manufacture of many products, including plastic food and beverage containers, thermal receipt papers, the lining of some food cans, and other products. BPA can migrate into foods and beverages from food packaging and reusable containers. Because BPA is used in diverse consumer and commercial products, it is ubiquitous in the environment. As a result, detectable levels of BPA and/or its metabolites in human serum, saliva, urine, amniotic fluid, and breast milk have been reported. The estimated 95th–97th percentile of typical daily aggregate exposure to BPA is <0.6–1.5 μg/kg body weight (bw)/day for adult humans and 0.3–1.1 μg/kg bw/day for children and teenagers (WHO 2011).

BPA has been shown to bind to both nuclear and cell membrane estrogen receptors. At higher exposure levels, BPA is an androgen receptor antagonist and interacts with other nuclear receptors, including the glucocorticoid receptor, peroxisome proliferator-activated receptor gamma, and thyroid hormone receptor (Delfosse et al. 2014; MacKay and Abizaid 2018; Sheng et al. 2019). In addition, it has been reported that BPA at micromolar concentrations may also

exert its action on GnRH neurons via the nuclear receptor transcription factor estrogen-related receptor gamma and other pathways, including voltage-gated sodium channels (Klenke et al. 2016). Although BPA can have hormone-like effects among other reported effects [e.g., Hunt et al. (2003)], whether BPA can exert adverse effects at human exposure levels remains a point of contention. Many studies of experimental animals and human research have reported an association of BPA exposure with a variety of health problems including infertility, weight gain, behavioral changes, early-onset puberty, prostate and mammary gland cancers, cardiovascular effects, and diabetes (Rochester 2013; Vandenberg 2014; Vom Saal and Vandenberg 2020). BPA has been identified as a substance meeting the WHO/IPCS definition of an endocrine disruptor by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) (reviewed in Beausoleil et al. (2018). The European Chemicals Agency listed BPA in the Candidate List of Substances of Very High Concern (SVHC) due to its reproductive toxicity properties (ECHA 2017a) and identified BPA as a SVHC because of its endocrine disrupting properties causing probable serious effects to the environment and human health (ECHA 2017b; 2017c). In contrast, the European Panel on Food Contact Materials, Enzymes, Flavorings and Processing Aids assessed the risks to public health associated with BPA exposure and concluded there was no health concern for any age group from dietary exposure and low health concern from aggregated exposure (EFSA 2015). The U.S. Food and Drug Administration (FDA) released a series of comprehensive reviews of more than 300 scientific studies evaluating low doses of BPA (U.S. FDA 2011; 2012; 2014a; 2014b). In its 2014 review of the literature, the FDA concluded that, “Following our extensive review of ‘low-dose’ BPA literature, we are unable to construct a plausible or logical comprehensive toxicological profile or explanation for the many claimed effects of BPA, largely due to the inconsistencies that currently exist within this literature” (U.S. FDA 2014b).

CLARITY-BPA Program

In 2010, the National Toxicology Program (NTP) and National Institute of Environmental Health Sciences (NIEHS) developed a new guideline-compliant study, conducted in accordance with GLP, to reconcile uncertainties in the scientific literature on the potential for toxicity of lower dose levels of BPA. Specifically, this chronic toxicity study would examine a wide dose range of BPA (including the lower dose range tested in many investigational research studies), use a long-term dosing protocol that encompassed developmental exposure, administer BPA via a human-relevant oral dose route, and assess additional endpoints not typically assessed in guideline-compliant studies. In addition to the periodic comprehensive reviews of BPA literature and safety assessment by the FDA, this research effort would offer an opportunity to test a new, collaborative research model based on enhancing the links between investigational and guideline-compliant research and possibly provide the risk assessment community a more comprehensive body of research to inform decision-making.

In response, NIEHS and FDA developed a consortium-based research program under the umbrella of NTP to link guideline-compliant research and investigational research. This initial proof-of-concept collaboration was called the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA), and it used BPA as a test chemical (Schug et al. 2013). The study design of the CLARITY-BPA program was structured to reduce many potential sources of bias. These study design features included such things as randomized allocation of animals to the study, use of a single pup per sex per litter for a given endpoint to control for litter

effects, monitoring the purity of BPA and accuracy of dosing, quantification of the background dietary intake of BPA, and blinding of the treatment group of the biological samples, among others. The CLARITY-BPA program combined (1) a "core" study, a rat perinatal exposure guideline-compliant 2-year chronic toxicity study of BPA, with (2) an investigational research arm conducted by 14 university-based researchers funded for this purpose. The 14 grantees were selected by the NIEHS Division of Extramural Research and Training from responses to an open competitive request for application (RFA-ES-10-009) to collaborate with NIEHS and FDA in the overall study design and to conduct investigations using biological samples and animals from the core study. The selected grants were reviewed by NTP for feasibility of the proposed experimental design only. Thus, the CLARITY-BPA program, initiated and funded by NIEHS through NTP with the participation of FDA and grantee investigators, represents a new model for research.

About the CLARITY-BPA Compendium Report

This report summarizes the experimental procedures, findings, and authors' interpretations in each of the original peer-reviewed publications from the CLARITY-BPA research program. The findings from the core study presented in the following chapters include all measurements found significant ($p < 0.05$) by the statistical tests applied, which in the case of histopathology data did not include adjustment for multiple comparisons. The report is organized into 10 chapters by organ or organ system, including brain and behavior, cardiac, immune, mammary gland, ovary, penile function, prostate gland and urethra, testis and epididymis, metabolism and thyroid hormone, and uterus. The reader may refer to the raw data in the NTP's Chemical Effects in Biological Systems (CEBS) database (https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA) and study details in the original peer-reviewed publications for more information. In addition, Camacho et al. (2019) presented a summary of the core study data but included a more expansive discussion of the FDA's conclusions. An integrated analysis of findings from eight CLARITY-BPA grantee studies has also been published (Heindel et al. 2020); however, this paper is not summarized in this report.

Several grantee studies remain unpublished; the designs of these studies are briefly described in the following chapters, but their findings are not included. All data from the core study and the majority of data from the grantee studies, including unpublished studies, are available in the NTP CEBS database.

Effort has been made to provide an accurate account of the experimental procedures, findings, and authors' interpretations reported in each of the peer-reviewed publications. No attempt was made to reconcile any differences in authors' interpretations in common tissues or to reanalyze published data. For complete details of these studies and the authors' conclusions, please refer to the original peer-reviewed publications.

Methods

The initial draft of the final report, including study summaries, was prepared at NIEHS by a team of investigators in the Office of Health Assessment and Translation, Division of NTP. To support the summaries of the Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity (CLARITY-BPA) program, manual data extraction from the core study and investigational research studies was conducted, and extracted data were presented in tables by the contract staff for NIEHS. Data extraction was performed by one member of the evaluation team and checked by a second member for completeness and accuracy. If needed, discrepancies in data extraction were resolved by discussion or consultation with additional members of the evaluation team. Data extraction from studies was limited to information used to support the summaries in each chapter. In general, each chapter presents two tables—one table with endpoints measured and timing of assessment for included studies and a second table with endpoint-specific results for statistically significant data. For study data presented in figures, members of the evaluation team contacted authors of studies to obtain data from figures that were considered important for evaluating key study findings. Following completion (including quality assurance/quality control) of all tables for individual chapters, global consistency checks were performed to ensure an accurate and consistent presentation of study results across chapters. The draft organ-specific chapters were shared for comment and revisions with staff at the FDA National Center for Toxicological Research (NCTR) and the grantee responsible for each organ/system. Drafts of the Executive Summary were shared with all CLARITY-BPA participants for review and comment.

Detailed materials and methods for the core study and the investigational studies are contained in the specific study reports and publications cited in each chapter. Because the core study was the source of animals for all of the investigational studies, a brief abstract of the study methods is given here. Further experimental details can be found in *NTP Research Report on the CLARITY-BPA Core Study: A Perinatal and Chronic Extended-Dose-Range Study of Bisphenol A in Rats* (NTP 2018) and in Heindel et al. (2015).

Rats (Sprague Dawley/CD23/NctrBR [NCTR-SD]) were obtained as weanlings from the NCTR breeding colony and placed under study conditions (soy- and alfalfa-free diet 5K96 [Lab Diet, St. Louis, MO] in polysulfone cages with hardwood chip bedding, glass water bottles, and food-grade silicone stoppers). Study materials were monitored for background BPA levels; the only material with detectable levels of BPA was the diet, which had less than 3 ppb BPA.

BPA was purchased from TCI America (Portland, OR; product #B0494, lot #6052012) and verified by NTP contractors to be 99.9% pure. BPA, formulated in 0.3% carboxymethylcellulose (CMC), was administered by oral gavage to pregnant rats from gestation day (GD) 6 through the start of labor and then by oral gavage to the pups, at the same dose (μg/kg bw/day) given to the dams from postnatal day (PND) 1 (day of birth = PND 0) until termination. Treatment groups included 0 (CMC vehicle control), 2.5, 25, 250, 2,500, and 25,000 μg BPA/kg bw/day. CMC (sodium salt; product #C5013, lot #041M0105V) was obtained from Sigma-Aldrich (St. Louis, MO). The low dose selected, 2.5 μg BPA/kg bw/day, provided a margin of exposure at least 10-fold higher than the maximum allowed background dietary exposure in the animal feed, while the high dose of 25,000 μg BPA/kg bw/day was viewed as providing an adequate margin of human exposure, greater than 25,000-fold based on the aggregate human exposure estimates of

<0.5 µg BPA/kg bw/day as mentioned above. Dose formulations were periodically certified to be within 10% of the target dose and were used within their established stability window. In addition to animals that were dosed daily throughout the study, the core study also included a stop-dose arm with animals dosed daily until PND 21 and then maintained without sham gavage or other further dosing until termination to assess any potential BPA effects that were due to early exposure only. Because many of the effects of BPA reported in the literature are associated with estrogen signaling pathways, two dose groups of the orally active estrogen ethinyl estradiol (EE2; administered at 0.05 and 0.5 µg/kg bw/day) were also included in the continuous-dose arm to assess the sensitivity of the test system to an estrogen. Females were singly housed during gestation and with their litters following delivery until PND 21.

At weaning on PND 21, one animal per sex per litter was assigned to each of the following study arms of the core study (Figure 1):

1) Continuous dosing to sacrifice at 2 years (continuous-dose terminal sacrifice, 46–50 animals per sex per vehicle control or BPA treatment group and 26 animals per sex per EE2 group)
2) Continuous dosing to sacrifice at 1 year (continuous-dose interim sacrifice, 20–26 animals per sex for all groups)
3) No further treatment after PND 21 until sacrifice at 2 years (stop-dose terminal sacrifice, 46–50 animals per sex per preweaning vehicle control or BPA group)
4) No further treatment after PND 21 until sacrifice at 1 year (stop-dose interim sacrifice, 20–26 animals per sex for preweaning vehicle control and BPA groups)

The investigational studies used siblings from the core study animals, which were housed and dosed under the same conditions. Animals from the core study assigned to the investigational studies were sacrificed at PND 1, PND 15, PND 21, PND 90, 6 months, or 1 year of age. The number, sex, dosing arm (continuous or stop, after weaning), and sacrifice times, as well as other design elements (e.g., restrictions on litter size and sex ratios) used in each investigational study, were determined by the grantee and each request was matched to the extent possible by NCTR. In some cases, insufficient numbers of pups were produced to provide all the animals requested by the grantees. In these cases, some pups were reallocated from the core study to grantee studies to partially mitigate this shortfall. The sacrifices were conducted at NCTR, and tissues were isolated and sent to the respective grantee laboratories following the instructions provided a priori by each grantee to NCTR. An effort was made to collect tissues for as many investigational studies as possible from the same animals to minimize animal usage. For the behavior tests, grantees traveled to NCTR to conduct the tests, which followed pre-agreed protocols; tissues were then collected from these animals at NCTR for later evaluation at the grantees' laboratories. The penile function tests were conducted at NCTR by a grantee who also collected tissues to further evaluate in his laboratory.

Two additional sets of animals were generated at the request of two of the grantees. For the thyroid focus area study, a subset of animals was dosed via drinking water with propylthiouracil to assess the responsiveness of the experimental system to alterations in thyroid hormone status. For testis function and sperm profiles, the investigational study by Dere et al. (2018) also included a 250,000 µg BPA/kg bw/day treatment, which was given by daily oral gavage to pregnant dams at NCTR from GD 6 through the start of labor and to pups from PND 1 until they

were sacrificed at PND 90. Following the start of the CLARITY-BPA in-life phase of the study, analysis of serum samples collected for internal dosimetry assessments in a 90-day pre-chronic study performed at NCTR utilizing BPA doses as high as 300,000 µg/kg bw/day, indicated that serum from control rats was found to contain BPA glucuronide at levels comparable to those found in the 2.5 µg BPA/kg bw/day dose group, suggesting contamination from an unidentified source (Churchwell et al. 2014; Delclos et al. 2014). Although similar "contamination" was not exhaustively investigated in the CLARITY-BPA study (Heindel et al. 2015), it thus was assumed possible. For that reason, steps were taken to individually track and evaluate animals for the core and grantees' studies that were housed in the same room as the animals receiving the 250,000 µg BPA/kg bw/day dose. When this occurred, statistical analyses that include or omit the animals co-housed with the 250,000 µg BPA/kg bw/day dose group are provided in the various chapters. The results of these sensitivity analyses that excluded the co-housed animals were only reported if they resulted in a change in an "adverse" direction for histopathology, that is, reduced incidences in the sensitivity analysis that were not significant in the inclusive tests were not reported.

With respect to analysis, there were several statistical approaches applied to the core study data, and these are outlined in the respective study chapters and in the core study report (NTP 2018). Statistical analyses of the histopathology data were conducted for any lesion that was diagnosed in two or more animals in any dose group in the interim sacrifice groups or four or more animals in the control and BPA groups at the terminal sacrifice. Lesion incidences in interim sacrifice animals were analyzed by the Cochran-Armitage trend test and Fisher's exact (CAFE) tests to compare the trend across dose groups and the pairwise comparisons in each dose group to the vehicle control. Lesion incidences in terminal sacrifice animals were analyzed by the Poly-3 test, which adjusts for intercurrent mortality. Two secondary statistical tests were applied to incorporate severity scores in addition to lesion incidences—the Jonckheere-Terpstra trend test/Shirley-Williams pairwise comparison (JT/SW) test, which enforces an assumption of a monotonic response, and the relative treatment effect (RTE) test, which does not enforce monotonicity. Neither the JT/SW nor RTE test adjusts for survival.

In the core study, statistical outcomes were adjusted for multiple comparisons across dose groups for each endpoint with the exception of histopathology. For histopathology data, single-sided p values were used, and no adjustments were made for multiple comparisons. No corrections for the multiplicity of endpoints measured in the study were made in any case. This approach is standard for NTP chronic studies.

Additional statistical approaches were used by individual grantees, and these are also outlined in detail in their respective publications. All statistically significant findings reported in the publications are reflected in the tables and text in each outcome chapter.

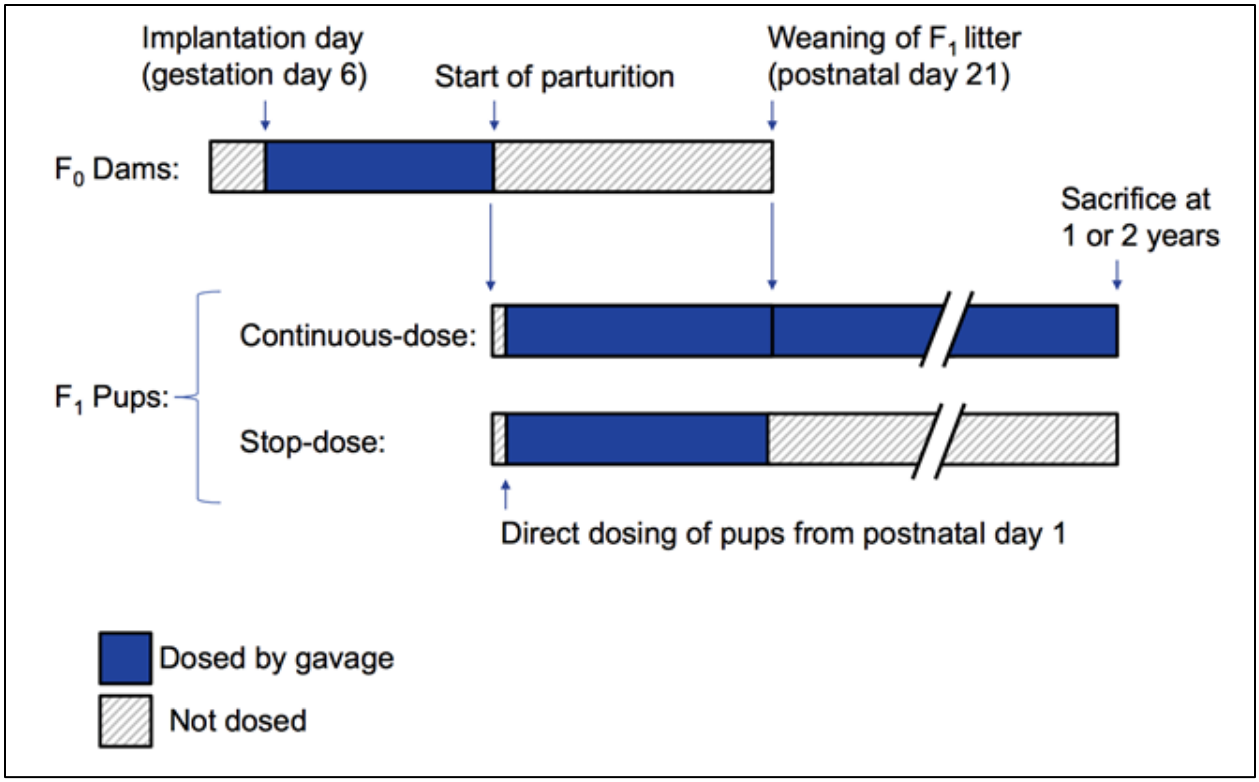


Figure 1. CLARITY-BPA Core Study Design

The core study began with five partial study loads from September through December 2012, with in-life study termination from October 2014 through January 2015.

Results

Chapter 1. Brain and Behavior

Findings of the Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity (CLARITY-BPA) core study (NTP 2018) and the investigational studies by the laboratories of Heather Patisaul (Arambula et al. 2016; Arambula et al. 2017; Arambula et al. 2018; Rebuli et al. 2015; Witchey et al. 2019) and Cheryl Rosenfeld (Cheong et al. 2018; Johnson et al. 2016) with respect to the potential effects of bisphenol A (BPA) on the brain and behavior are summarized below. All individual animal data for these studies are available online (https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA) with exception of two studies. The data for the remaining two studies of brain and behavior that evaluated tissues from the CLARITY-BPA program, but were not funded by the program, are available upon request from Dr. Heather Patisaul (Arambula et al. 2018; Witchey et al. 2019). The study details, including endpoints measured and timing of assessment, are detailed in Table 2. The statistically significant findings from each investigational study and the statistically significant findings from the core study on related endpoints are included in Table 3.

Study Summaries

Core Study: NTP (2018)

Methods

The endpoints evaluated in the core study included brain weights at 1 year and histopathology of the brain, spinal cord, and peripheral nerves at 1 year and study termination (Table 2). Clinical observations were recorded weekly or when a significant clinical observation was observed. There were no formal behavioral assessments.

Findings

There were no significant findings reported for brain weights, clinical observations related to behavior, or increases in histopathological findings in any BPA dose group in either the stop- or continuous-dose study arm. There were decreases in incidence in some BPA dose groups of brainstem compression and other lesions that are typically considered artifacts occurring during necropsy (NTP 2018).

Ethinyl estradiol

For continuous-dose-arm EE2-exposed animals, brain stem compression and hemorrhage, and ventricular dilatation of the cerebrum were observed for some animals, but as stated above, these observations are considered artifacts and were not discussed in NTP (2018).

Author conclusions

The authors did not provide specific conclusions concerning the effects of BPA exposures on the brain or behavior, but stated that, overall, the findings were not "dose responsive, sometimes occurring in only one low or intermediate dose group, and did not demonstrate a clear pattern of consistent responses within or across organs within the stop- and continuous-dose arms and the interim and terminal sacrifices" (NTP 2018).

Investigational Study: Rebuli et al. (2015)

Methods

Pups from dams dosed with 2.5, 25, or 2,500 μg BPA/kg bw/day or vehicle control (n = 11–12 per group) from GD 6 to PND 21 were weaned on PND 21 and then moved to another building on the NCTR campus where they were subjected to several behavioral tests. Over the course of 4 days, the light cycle was modified to accommodate behavioral testing during the dark phase. Juveniles were tested from PND 25 to PND 27, and adults were tested similarly either from PND 97 to PND 111 or PND 111 to PND 125, accounting for estrous cycle stage. Behavioral tests were selected with high predictive value for anxiety and included the elevated plus maze (EPM), open field (OF), and zero maze (adults only) (Table 2).

Findings

Juvenile testing showed few significant effects of BPA exposures on behavioral outcomes. Sex differences between male and female controls were observed, but in some cases the specific behaviors were in the opposite sex when compared with other studies with Sprague Dawley rats. Similar to the juveniles, there were no consistent effects of BPA exposures observed in adult rats of either sex. Sex-specific differences were found with the EPM and OF tests in the adult rats, and the results were stated to be in agreement with prior reported findings with the NCTR-SD rat.

Ethinyl estradiol

Similar behavioral measures with the high dose of EE2 (0.5 μg/kg bw/day) also did not demonstrate differences from controls. Some findings from the EPM test showed differences in comparisons between the EE2 group and BPA-dosed groups.

Author conclusions

The authors concluded that evidence for BPA-related effects was inconsistent and not indicative of a biologically meaningful effect on anxiety or exploratory behavior. Post hoc power calculations suggested that the group size of 12 animals was only moderately powered to detect an effect, if present. The authors suggested that the light phase change during PND 21–25 and gavage dosing may have caused stress that might have masked BPA-related effects on anxiety and locomotion.

Table 2. Study Details and Summary of Measured Brain and Behavior Endpoints

Endpoint Category	Specific Measure	Arambula et al. (2016)[1]	Arambula et al. (2018)[1]	Rebuli et al. (2015)[2]	Arambula et al. (2017)[3]	Witchey et al. (2019)[3]	Johnson et al. (2016)[4]	Rebuli et al. (2015)[2]	Cheong et al. (2018)[5]		NTP (2018)[6]	
		Age at Assessment										
		PND 1	PND 1	PND 25–27	PND 28	PND 28	PND 97 or 111	PND 97–125	PND 101–107 or 115–119	PND 101 or 115	1 yr	2 yr
Behavior[7]	Anxiety	–	–	□	–	–	–	□	–	–	– –	– –
	Spatial navigational learning and memory	–	–	–	–	–	□	–	–	–	– –	– –
Morphology[8]	Brain nuclei	–	–	–	□	–	–	–	–	–	– –	– –
DNA Methylation[9]	Hippocampus	–	–	–	–	–	–	–	□	□	– –	– –
	Hypothalamus	–	–	–	–	–	–	–	□	□	– –	– –
	Hippocampus, correlation between DNA methylation and behavior	–	–	–	–	–	–	–	□	□	– –	– –
Gene Expression[10]	Amygdala	–	●	–	–	–	–	–	–	–	– –	– –
	Hippocampus	●	–	–	–	–	–	–	□	□	– –	– –
	Hypothalamus	●	–	–	–	–	–	–	□	□	– –	– –
	Hippocampus, correlation between gene expression and behavior	–	–	–	–	–	–	–	□	□	– –	– –
Organ Weight[11]	Brain weight	–	–	–	–	–	–	–	–	–	● □	– –
Hormone Function[12]	Hypothalamus	–	–	–	–	□	–	–	–	–	– –	– –
Pathology[13]	Brain stem	–	–	–	–	–	–	–	–	–	● □	● □

Endpoint Category	Specific Measure	Arambula et al. (2016)[1]	Arambula et al. (2018)[1]	Rebuli et al. (2015)[2]	Arambula et al. (2017)[3]	Witchey et al. (2019)[3]	Johnson et al. (2016)[4]	Rebuli et al. (2015)[2]	Cheong et al. (2018)[5]		NTP (2018)[6]	
		Age at Assessment										
		PND 1	PND 1	PND 25–27	PND 28	PND 28	PND 97 or 111	PND 97–125	PND 101–107 or 115–119	PND 101 or 115	1 yr	2 yr
	Cerebrum	–	–	–	–	–	–	–	–	–	● □	● □
	Peripheral nerves	–	–	–	–	–	–	–	–	–	● □	● □
	Spinal cord	–	–	–	–	–	–	–	–	–	● □	● □

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.
yr = year; bw = body weight; PND = postnatal day.
[1]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose BPA treatments. Animals were sacrificed at PND 1 and examined for effects.
[2]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, or 2,500 µg/kg bw/day using stop-dose BPA treatments. Animals were examined for effects as juveniles (PND 25–27) and adults (PND 97–111 or PND 111–125).
[3]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, or 2,500 µg/kg bw/day using stop-dose BPA treatments. Animals were sacrificed at PND 28 and examined for effects.
[4]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, or 2,500 µg/kg bw/day using stop-dose BPA treatments. Animals (males, females, or males and females combined) were examined for effects beginning at PND 90 or PND 104 for 7 consecutive days (i.e., through PND 97 or 111).
[5]Male and female Sprague Dawley rats were exposed via gavage at 2,500 µg/kg bw/day using stop-dose BPA treatments. Animals were examined for effects beginning at PND 101–107 or 115–119 (females) or at PND 101 or PND 115 (males).
[6]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr or 2 yr and examined for effects.
[7]Rebuli et al. 2015 endpoint list: elevated plus maze: distance traveled in open arms, entries in open arms, stretch attends, time in open arms; open field: distance traveled, entries into center, time in center (examined at all time points); zero maze: distance traveled in open arms, stretch attends, time in open arms (examined at PND 97–125).
Johnson et al. 2016 endpoint list: Barnes maze: frequency of sniffing incorrect hole, likelihood to locate escape hole, search strategy, total distance traveled, velocity.
[8]Arambula et al. 2017 endpoint list: volume: anteroventral periventricular nucleus (AVPV), average medial amygdala (MePD), left medial amygdala (MePD), locus coeruleus (LC), right medial amygdala (MePD), sexually dimorphic nucleus (SDN).
[9]Cheong et al. 2018 endpoint list: correlation between *Bdnf* methylation and Barnes maze: rate of sniffing correct hole, total distance traveled, maze velocity; methylation: *Bdnf*, *Dnmt3b*, *Esr1*.
[10]Arambula et al. 2016 endpoint list: gene expression: *Esr1*, *Esr2*, *Oxt*, *Ptgds*, *Slc1a2*, *Slc32a1* (examined in hippocampus and hypothalamus) gene expression: *Lepr* (examined only in hypothalamus).
Arambula et al. 2018 endpoint list: gene expression: *Ar*, *Avpr1a*, *Camk4*, *Esr1*, *Esr2*, *Gadd45b*, *Grm5*, *Oxtr*.
Cheong et al. 2018 endpoint list: gene expression: *Ar*, *Avp*, *Bdnf*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Esr1*, *Esr2*, *Oxtr*, *Oxt* (examined in hippocampus and hypothalamus).
Correlation between expression and Barnes maze behavior: rate of sniffing correct hole, velocity with *Avp*, *Bdnf*, *Dnmt3b*, *Esr2*, *Oxtr*, *Oxt*; total distance traveled and *Bdnf*.
[11]NTP 2018 endpoint list: brain weight: absolute, relative to body weight.
[12]Witchey et al. 2019 endpoint list: OXTR binding, *Oxt* and *Oxtr* expression in four brain regions: dorsolateral bed nucleus of the stria terminalis (BNSTdl), posterior bed nucleus of stria terminalis (BNSTp), paraventricular hypothalamic nucleus (PVN), and ventromedial hypothalamus (VMH).
[13]NTP 2018 endpoint list: comprehensive assessment of neoplastic and nonneoplastic lesions (e.g., brain stem: compression, hemorrhage; cerebrum: benign granular cell tumor, hemorrhage, ventricle dilatation; histological abnormalities: peripheral nerves, spinal cord).

Investigational Study: Johnson et al. (2016)

Methods

Using a separate set of animals similarly handled as pups in Rebuli et al. (2015), adult males and females (n = 12) were tested for effects on spatial navigation in the control, 2.5, 25, and 2,500 µg BPA/kg bw/day stop-dose groups. Spatial navigation was assessed beginning on day 90 or 104 for 7 days using a modified Barnes maze apparatus, and learning was assessed over the 7-day test period by the change in time required to locate the escape hole, with additional measures for error rate, distance traveled, velocity, and search strategy (Table 2). Serum testosterone levels, reported here, were measured in pups on PND 25–27 after completion of the tests performed by and described in Rebuli et al. (2015).

Findings

Results showed generally expected improvements in spatial navigation performance across the 7-day period for both BPA-exposed and control groups. The overall likelihood ratio of exposed groups finding the correct escape hole was lower (indicating a longer latency) for females exposed to 2,500 µg BPA/kg bw/day, and lower but not statistically significant, for the 2.5 µg BPA/kg bw/day group (Figure 2; Table 3). Conversely, 2.5 µg BPA/kg bw/day males had an increased likelihood of finding the correct escape hole (decreased latency) compared to controls. There were no differences noted in measures of search strategy, meaning that all groups employed direct versus inefficient strategies at comparable percentages. Serum testosterone levels in these animals showed expected sex differences but were not affected by BPA exposure.

Ethinyl estradiol

Similar behavioral measures with the high dose of EE2 (0.5 µg/kg bw/day) did not demonstrate differences from controls.

Author conclusions

The authors concluded the current findings indicate that developmental exposure to BPA can disrupt aspects of spatial navigational learning and memory in a sex-dependent manner. The 2,500 µg BPA/kg bw/day, and to a lesser extent the 2.5 µg BPA/kg bw/day dose, prolonged latency in females, and the latter dose improved responses in males. The authors also noted that BPA exposures to the F_0 females might have adversely affected their parenting behaviors, as a possible study limitation, although parenting behavior was not measured in these studies.

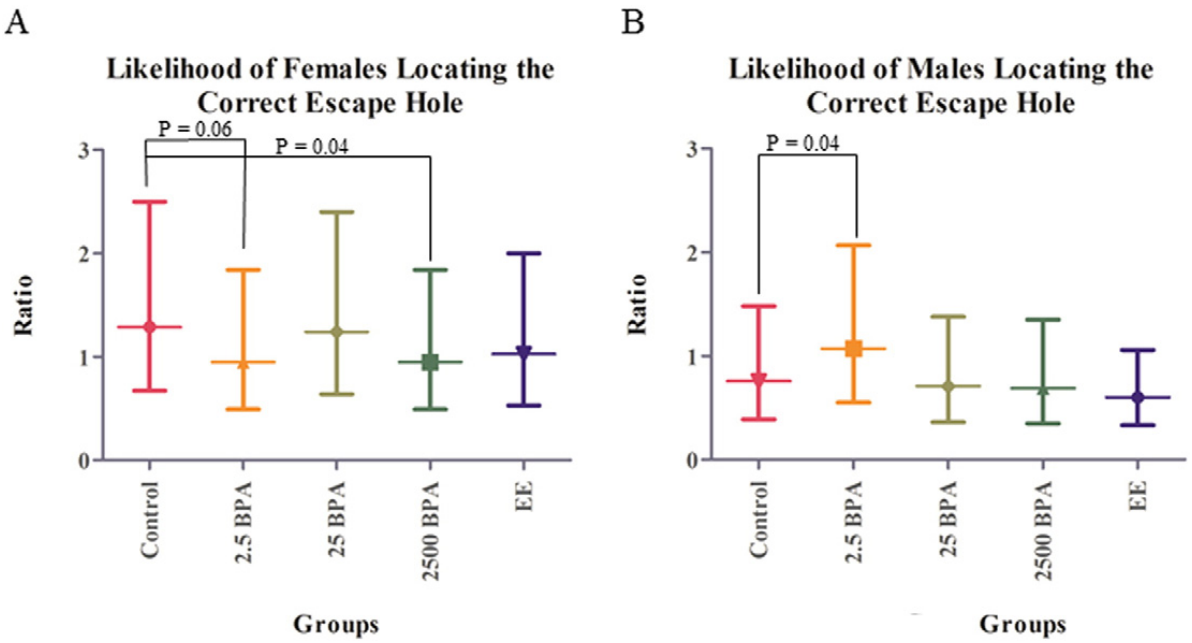


Figure 2. Overall Ratio for Female and Male Rats in Each Treatment Group to Locate the Escape Hole

Note that higher ratios indicate shorter latency. For both panels, the upper, middle, and lower bars represent the ratio of locating the correct escape hole at the 95% upper confidence limit, mean, and 95% lower confidence limit, respectively, for each group. Comparisons of the significant two-way interaction for treatment by sex are shown. (A) Overall ratio for females. (B) Overall ratio for males. [Reprinted with permission from Johnson et al. (2016).]

Investigational Study: Cheong et al. (2018)

Methods

Following evaluation in the Barnes maze, the adult animals from the studies described in Johnson et al. (2016) were sacrificed, and their brains were isolated and shipped on dry ice to the Rosenfeld lab. Males were taken on PND 101 or PND 107, and females were taken when in estrus between PND 101 and PND 107 or between PND 115 and PND 119. The only BPA dose groups evaluated were at 2,500 µg/kg bw/day, as the 2,500 µg/kg bw/day females showed a significant increased latency in the Barnes maze. Hippocampal and hypothalamic regions were micro-punched, and DNA and RNA were isolated from five animals per group. Gene expression was analyzed by quantitative real-time PCR (qRT-PCR) for *Dnmt3a, Dnmt3b, Dnmt1, Esr1, Esr2, Avp, Ar, Oxtr, Oxt, and Bdnf,* genes shown in prior studies to be affected by BPA (Table 2). DNA methylation patterns of the putative 5' promoter regions of *Bdnf* in the hippocampus and *Esr1* and *Dnmt3b* in the hypothalamus were assessed by bisulfite sequencing. Gene abbreviations are defined in the section below and in Appendix A of this report.

Findings

Of the 10 genes examined, only *Avp* (arginine vasopressin) expression was significantly altered (upregulated) in the hippocampus in the 2,500 µg BPA/kg bw/day group of females (Table 3). In the BPA-dosed males there was a nonsignificant increase in *Oxtr* (oxytocin receptor) expression in hippocampus and a significant decrease in hypothalamic expression of *Dnmt3b* (DNA methyltransferase 3 *beta*), *Bdnf* (brain-derived neurotropic factor), *Esr1* (estrogen receptor

alpha), and *Oxtr* in hypothalamus of males exposed to BPA compared to controls. BPA exposure diminished or reversed some of the sexually dimorphic patterns of gene expression observed in male versus female controls. With respect to effects of promoter region DNA methylation, BPA exposure was associated with hypermethylation of the *Bdnf* promoter region in the hippocampus in females. Codon or site-specific changes in promoter region methylation were seen in *Dnmt3b* in BPA-exposed males and in *Esr1* in both BPA-exposed sexes in the absence of changes in overall methylation levels.

BPA exposure reversed or eliminated the sex-specific difference in expression patterns of *Avp, Oxt* and *Ar* in the hippocampus, and eliminated the sexual dimorphism in expression of *Dnmt3a, Dnm3b, Esr1, Esr2, Oxtr,* and *Bdnf* in the hypothalamus.

The authors explored correlations between hippocampal gene expression, DNA methylation, and behavioral changes in the Barnes maze (Figure 3). An "overall weak negative association" was noted for DNA promoter region methylation with gene expression of *Bdnf*, and there was a trend for a negative association for *Bdnf* expression and one measure of search strategy efficiency (sniffing the correct hole in the Barnes maze) in BPA-exposed females. The authors also reported an association between expression levels of five genes (either positively or negatively) with velocity or sniffing the correct hole in the Barnes maze test in control animals. BPA exposure was reported to negate these associations.

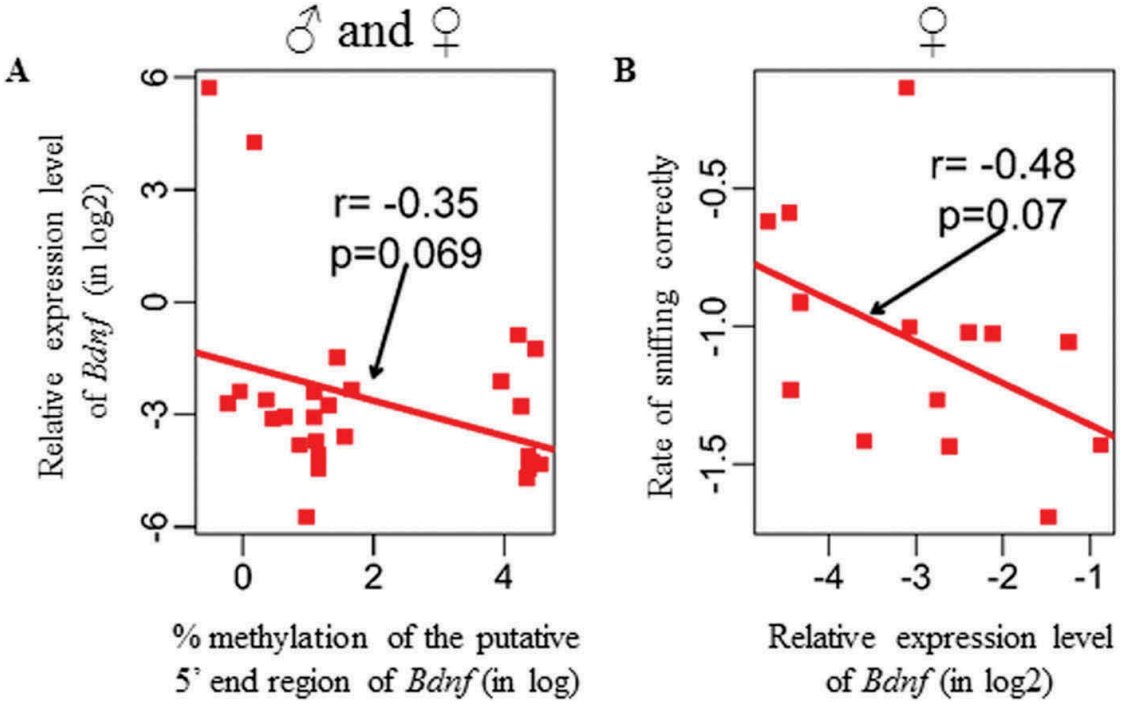


Figure 3. Correlation Analyses for Hippocampal *Bdnf* Gene Expression in Young Adult Rats Following Developmental Exposure to Vehicle Control

(A) Overall correlation between the expression level of *Bdnf* and % promoter methylation of *Bdnf* in rats with all data points included. (B) Overall correlation between the expression level of *Bdnf* and the rate of sniffing the correct hole in Barnes maze (citation) in female rats. Gene expression levels are expressed in log 2 values and as Ct value of Rpl19 relative to that of the target gene. Percentage methylation of the putative 5' end region of *Bdnf* is in log value. $P < 0.05$ was considered as statistically significant. Only samples with a detectable Ct value were used in the correlation analysis. [Reprinted with permission from Cheong et al. (2018).]

Ethinyl estradiol

In similar gene expression studies with 0.5 µg/kg bw/day EE2-exposed animals, in the female hypothalamus there was a 3.8-fold greater expression in *Dnmt3b*, and in the male hypothalamus there was a 5.6-fold decrease in both *Dnmt3b* and *Bdnf* expression, and *Esr1* expression was 11-fold lower compared to controls. These changes were consistent in direction with the gene expression changes in BPA-exposed males. However, *Esr2* was also expressed at a 3.7-fold lower level in the EE2 group of males and was not affected in the BPA-exposed males. EE2 was also found to eliminate the sex-specific differences in gene expression of *Dnmt3a*, *Dnmt3b*, *Dnmt1*, *Esr1*, *Esr2*, *Ar*, *Oxtr*, and *Bdnf* in the hypothalamus. EE2 exposure resulted in hypomethylation of the promoter region of *Bdnf* from the hippocampus of females compared to controls.

Author conclusions

The authors interpreted the findings to indicate that "BPA exposure induced non-EE-like gene expression and epigenetic changes in adult rat hippocampi, a region involved in spatial navigation."

Investigational Study: Arambula et al. (2017)

Methods

Utilizing the pups described in Rebuli et al. (2015), following the completion of behavioral testing on PND 27, control, 2.5, 25 and 2,500 µg BPA/kg bw/day exposed animals were sacrificed on PND 28; the brains were removed, frozen on dry ice and shipped to the Patisaul lab. Brains were cryosectioned and stained for Nissl substance to visualize anatomical structures. Unbiased stereology was performed to determine volumes of the sexually dimorphic nucleus (SDN), the anteroventral periventricular nucleus (AVPV), the posterodorsal portion of the medial amygdala (MePD), and the locus coeruleus (LC) (Table 2).

Findings

As expected, all areas except the LC showed sexually dimorphic volumes when comparing male and female control animals. There were no effects of BPA exposure on these sex differences; however, for all BPA-exposed female groups (2.5, 25, and 2,500 µg BPA/kg bw/day) and for 25 and 2,500 µg BPA/kg bw/day exposed males, the volume of the AVPV was significantly larger than in the corresponding sex controls (Figure 4). Also, when evaluating the right MePD, females in the 2,500 µg BPA/kg bw/day exposure group showed a significantly larger volume, but this was not apparent in the left MePD, or when averaging the right and left MePD. No significant effects of BPA exposures were noted for the LC.

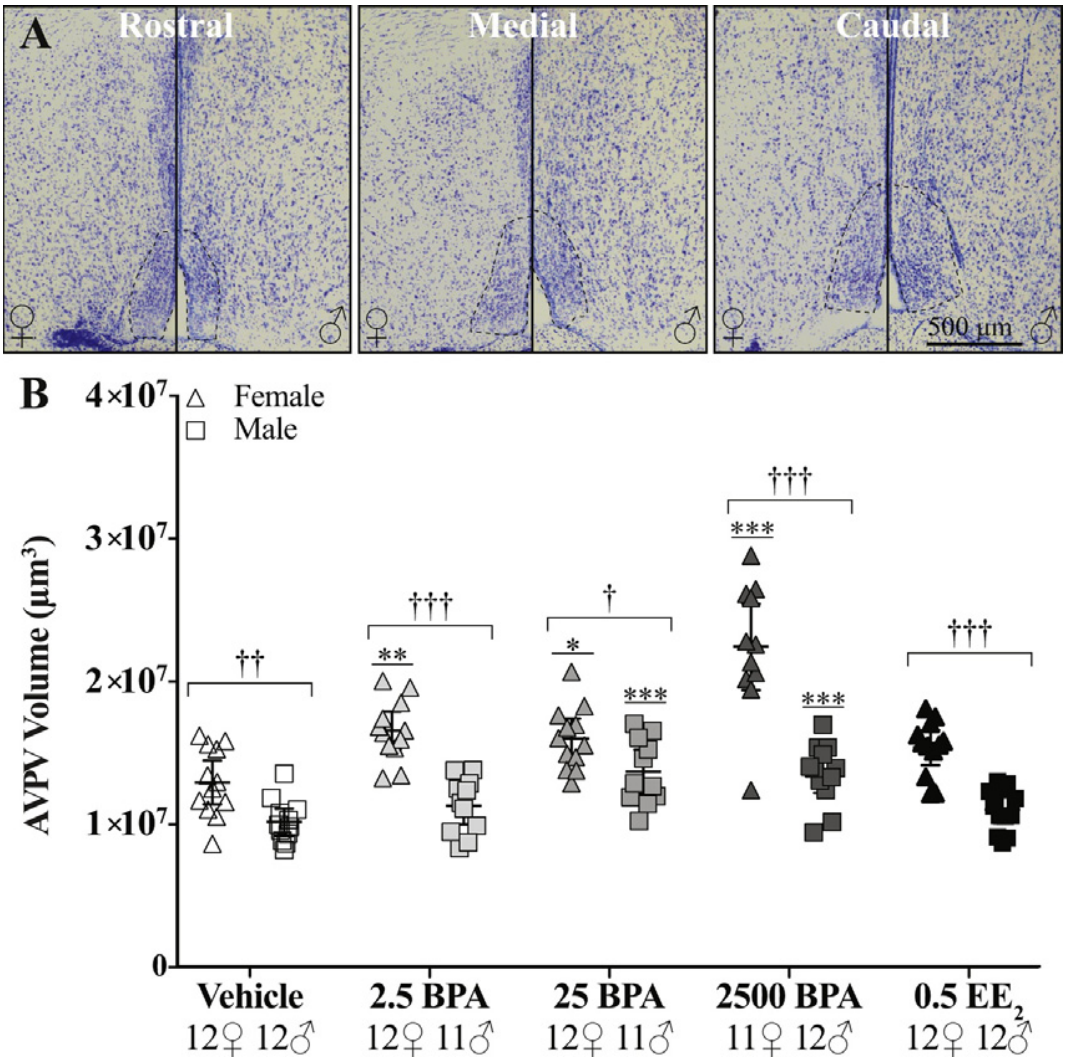


Figure 4. AVPV Volume in Rats at Age PND 28 Following Developmental Exposure to BPA or EE_2

(A) Representative thionin-stained coronal sections showing the anteroventral periventricular nucleus (AVPV). Sections are arranged from rostral to caudal and the dotted line indicates the boundaries of the area measured. In females, perinatal exposure to 2.5, 25, and 2,500 µg BPA/kg bw/day increased AVPV volume. (B) In males, perinatal exposure to 25 and 2,500 µg BPA/kg bw/day increased AVPV volume. As expected, AVPV volume was found to be significantly larger in females than in males in all exposure groups. Exposure to 0.5 µg EE2/kg bw/day had no significant effects on AVPV volume. Significant differences in volume compared to the same-sex vehicle group are represented by ***$p \leq 0.001$, **$p \leq 0.01$, and *$p \leq 0.05$. Significant sex differences in volume are represented by ††$p \leq 0.01$ and †$p \leq 0.05$. Error bars represent the 95% confidence interval and sample size is provided at the bottom. [Reprinted with permission from Arambula et al. (2017).]

Ethinyl estradiol

In similar studies with 0.5 µg EE2/kg bw/day, the only significant change from controls was an increase in volume of the locus coeruleus in males.

Author conclusions

The greater volume of the AVPV in response to BPA exposures in both sexes was suggested consistent with an antiestrogenic effect because endogenous estradiol masculinizes the AVPV, which diminishes its size. The AVPV influences sex-specific neuroendocrine and reproductive

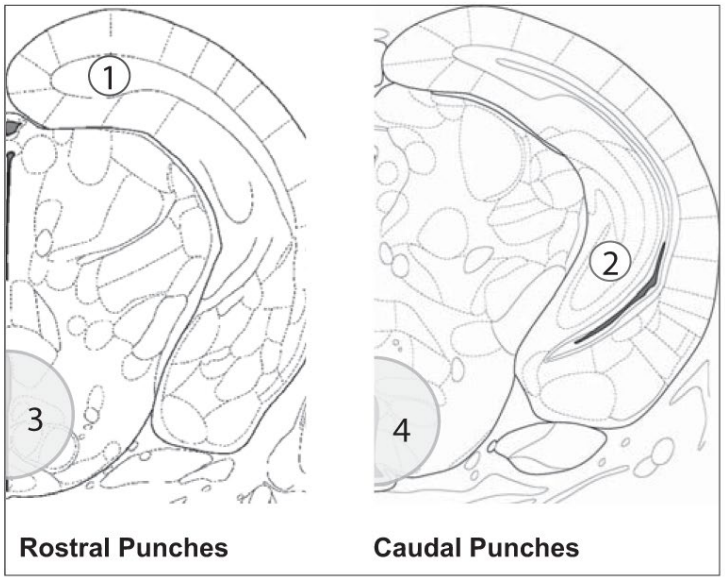
functions, including the preovulatory gonadotrophin surge (Simerly 2002; Wang and Moenter 2020).

Investigational Study: Arambula et al. (2016)

Methods

Pups from dams dosed with BPA or vehicle control from GD 6 were taken on PND 1 and decapitated, and the heads were flash frozen and sent to the Patisaul laboratory. Samples of hypothalamus and hippocampus were obtained by tissue punch to assess changes in full transcriptome expression or changes in specific genes (Figure 5). Punches from pups from the vehicle control, 2.5, and 2,500 µg BPA/kg bw/day dose groups were analyzed by RNA sequencing (RNA-seq) using an Illumina platform. Gene expression analysis by qRT-PCR was performed on tissues from all dose groups (2.5, 25, 250, 2,500, and 25,000 µg BPA/kg bw/day) and controls, with genes selected a priori because they were previously shown to be altered by BPA (*Esr1, Esr2*, and *Oxt*); reported to be sexually dimorphic in PND 1 hypothalamus (*Ptgds*); or identified from the RNA-seq data (*Lepr, Slc32a1*, and *Slc1a2*) (Table 2).

A



B

Groups	RNAseq	qRT-PCR
Vehicle	4♂, 4♀	6♂, 6♀ hyp 5♂, 6♀ hipp
2.5 BPA	4♂, 4♀	6♂, 6♀
25 BPA	0	5♂, 5♀
250 BPA	0	5♂, 5♀
2500 BPA	4♂, 4♀	6♂, 6♀hyp 6♂, 5♀ hipp
25000 BPA	0	5♂, 5♀
0.05 EE	0	5♂, 5♀
0.5 EE	0	5♂, 5♀

Figure 5. Location of Tissue Punches in Hippocampus and Hypothalamus and Number of PND 1 Rats Sampled for RNA-seq and qRT-PCR Following Developmental Exposure to BPA or EE2

(A) Anatomical representation of regions extracted via micropunch (obtained by approaching the regions of interest caudally and punching rostrally) and used for gene expression analysis. For each animal, 1 pair of bilateral anterodorsal hippocampal punches (1, unshaded) and 1 pair of bilateral caudoventral hippocampal punches (2, unshaded) were made, each 0.5 mm in diameter and 1.00 mm in depth. All 4 punches were combined, collectively comprising the whole hippocampus. Hypothalamic tissue consisted of 2 sequential punches (1.25 mm in diameter and 1.00 mm in depth): 1 anteromedial (3, shaded) and 1 caudomedial (4, shaded). (B) Sample sizes for RNA-seq and qRT-PCR. [Reprinted with permission from Arambula et al. (2016).]

Findings

There were few effects of BPA exposures on PND 1 hippocampal whole transcriptome gene expression. BPA exposure-related transcriptional changes were more numerous in the hypothalamus, and this effect was much more evident in males than in females. In 2.5 µg/kg bw/day BPA-exposed males, 639 genes were affected compared to controls by RNA-

seq analysis. At 2,500 µg BPA/kg bw/day, 1,107 genes had altered expression levels, and 371 affected genes were common to both BPA dose groups. Although many genes were statistically significantly affected, the fold change did not exceed ~1.6. Confidence in these findings was increased by demonstrating expected sexually dimorphic patterns of gene expression when comparing control males with control females. When assessed by qRT-PCR, *Esr1, Esr2*, *and Slc32a1* were markedly increased in females at 2.5, 250, and 25,000 µg BPA/kg bw/day, but not at 25 or 2,500 µg BPA/kg bw/day, and *Oxt* was increased at 2.5 and 250 µg BPA/kg bw/day (Table 3). *Oxt* was also significantly increased in the hypothalamus of 25 µg BPA/kg bw/day males compared to controls.

Ethinyl estradiol

In similar studies with 0.5 µg EE2/kg bw/day, qRT-PCR analyses indicated greater expression of *Esr2* in the hippocampus of males, and *Oxt*, *Slc32a*, and *Ptgds* expressions were greater in the hypothalamus of EE2 males and females than in controls.

Author conclusions

The authors stated that "the present studies found limited effects of BPA on the PND 1 rat hypothalamus and hippocampus but provide further evidence that developmental BPA exposure, at levels below the NOAEL, can alter brain mRNA levels of ERs and OT [*Oxt*]" (Arambula et al. 2016). A preferential effect on gene expression in the hypothalamus more so than the hippocampus was considered consistent with the hypothalamus' role in coordinating a wide range of neuroendocrine-related activities.

Investigational Study: Arambula et al. (2018)

Methods

Using the same tissues from the study described by Arambula et al. (2016), the Patisaul research group also evaluated gene expression changes in the amygdala on PND 1. Similar RNA-seq and qRT-PCR approaches were performed on tissue punches as in Arambula et al. (2016). Six genes were selected a priori for qRT-PCR based on their "(1) role in socioemotional behaviors, (2) sex-biased expression pattern in the amygdala, (3) sensitivity to BPA or estrogen and/or, (4) importance in sexual differentiation of the amygdala." These genes included *Ar, Avpr1a, Esr1, Esr2, Gadd4b*, and *Oxtr* (Table 2). One difference from the Arambula et al. (2016) study was that RNA-seq analyses were performed on the vehicle control, 25, and 250 µg BPA/kg bw/day groups instead of the 2,500 µg BPA/kg bw/day group. Additional genes were analyzed by qRT-PCR to validate the RNA-seq findings.

Findings

Full transcriptome expression analysis showed more affected genes in females than in males in the amygdala. There were 251 and 341 affected genes in 25 and 250 µg BPA/kg bw/day female dose groups, respectively, with 174 genes expressed in common between the dose groups. qRT-PCR was used to validate the RNA-seq findings for *Grm5* and *Camk4*, both of which were elevated in several of the female BPA dose groups and are involved in neurodevelopment (Table 3). Other qRT-PCR-based findings were higher expression levels of *Oxtr, Ar, CamK4* in the 25 µg BPA/kg bw/day dose group in males and *Esr2* in the 250 µg BPA/kg bw/day dose group in females.

Ethinyl estradiol

In similar qRT-PCR assessments of amygdala from animals receiving 0.5 µg EE2/kg bw/day, *Oxt, Camk4, Grm5*, and *Gadd45b* expressions were greater than in controls in females, and *Avpr1a* expression was greater in males. Also, expressions of *Camk4* and *Grm5* were greater than in controls in females receiving 0.05 µg EE2/kg bw/day.

Author conclusions

The authors interpreted these finding as supporting evidence that BPA affects the organization of oxytocin (*Oxt*) and arginine vasopressin neural systems, which have been shown to alter social behavior.

Investigational Study: Witchey et al. (2019)

Methods

Using brain tissues from stop-dose male and female pups described in Rebuli et al. (2015) (i.e., the residual brain tissues from these pups after the analysis in Arambula et al. (2017)), Witchey et al. (2019) carried out additional assessments of *Oxtr* binding across several brain regions. Receptor binding was analyzed in the bed nucleus of stria terminalis, dorsolateral (BNSTld), bed nucleus of stria terminalis, principle (BNSTp), paraventricular hypothalamic nucleus (PVN), and ventromedial hypothalamus (VMH).

Brain tissues were not available for all animals analyzed in Arambula et al. (2017), and the numbers of animals with sufficient tissues to analyze all four brain areas are indicated in Figure 6. Brains were cryosectioned, fixed in 0.1% paraformaldehyde, and stained with a cocktail containing the radionucleotide ^{125}I-ornithine vasopressin analog (^{125}I-OVTA), which is selective for the *Oxtr*. Films were incubated, developed, and analyzed for optical density.

Findings

Expected sex differences in *Oxtr* binding in control animals were found. *Oxtr* binding was greater in control males in the BNSTp, PVN, and VMN, and greater in control females in the BNSTld. Exposure to BPA resulted in significantly greater *Oxtr* binding in the BNSTld in males receiving 2.5 and 25 µg BPA/kg bw/day than in male controls (Table 3). Sex-specific differences in expression were lost in the BNST1d as well as the PVN and VMH in pups receiving all three dose levels of BPA (2.5, 25, and 2,500µ µg/kg bw/day) (Figure 6).

Ethinyl estradiol

In animals receiving 0.05 µg/kg bw/day of the control estrogen EE2, similar patterns were observed with dosing resulting in greater *Oxtr* binding in the BNSTld in males than in control males, and elimination of the sex differences in *Oxtr* binding between males and females in the BNSTld, PVN, and VMN.

Author conclusions

The authors stated that these findings represent "further evidence that perinatal exposure [to BPA] can affect brain sexual differentiation and the organization of the OT/OTR [*Oxt/Oxtr*] system" (Witchey et al. 2019).

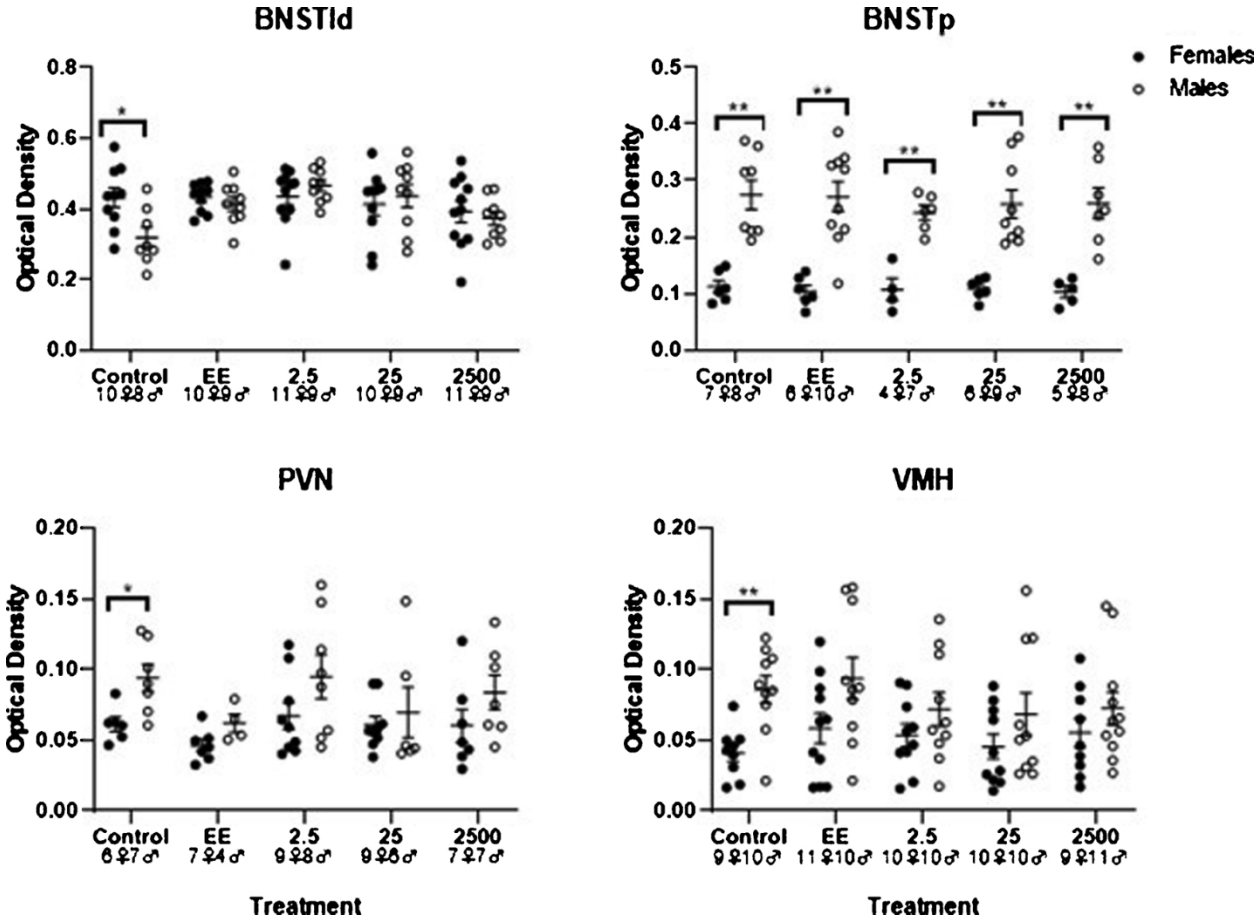


Figure 6. Expression Intensity of *Oxtr* Measured by Optical Density in the Four Regions Where Density Was Found to Be Sexually Dimorphic (BNSTld, BNSTp, PVN and VMH)

Male controls had significantly more *Oxtr* expression in the BNSTp, PVN and VMH. These sex differences were not statistically significant in the BNSTld, PVN or VMH in any of the exposed groups. No effect of exposure was found in females. The sample size for each region of interest is indicated below the exposure groups. Each dot represents a data point and bars are mean ± SEM. (*) $p \leq 0.05$ and (**) $p \leq 0.01$. [Reprinted with permission from Witchey et al. (2019).]

Table 3. Summary of Statistically Significant Brain and Behavior Endpoints

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
CD, PND 1	Gene Expression	***Ar* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	F	–	1 ± 0.21 [7]	0.92 ± 0.18 [6]	1.31 ± 0.27 [6]	1.84 ± 0.33* [6]	0.98 ± 0.34 [6]	1.76 ± 0.37 [6]	Arambula et al., 2018
		***Ar* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	M	–	1 ± 0.24 [6]	0.89 ± 0.29 [5]	2.43 ± 0.32** [7]	1.62 ± 0.18 [7]	0.69 ± 0.16 [5]	1.37 ± 0.27 [6]	Arambula et al., 2018
		***Avpr1a* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	M	–	1 ± 0.13 [6]	0.75 ± 0.13* [5]	2.27 ± 0.24** [7]	1.56 ± 0.13* [7]	0.86 ± 0.15 [5]	1.20 ± 0.18 [6]	Arambula et al., 2018
		***Camk4* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	F	–	1 ± 0.039 [5]	2.54 ± 0.49** [6]	2.02 ± 0.23** [6]	2.24 ± 0.28** [6]	1.68 ± 0.36 [6]	2.48 ± 0.37** [6]	Arambula et al., 2018
		***Camk4* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	M	–	1 ± 0.13 [5]	0.70 ± 0.21 [5]	1.75 ± 0.21** [7]	1.24 ± 0.12 [7]	0.62 ± 0.12 [5]	1.15 ± 0.17 [6]	Arambula et al., 2018
		***Esr2* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	F	–	1 ± 0.22 [7]	1.12 ± 0.14 [6]	1.30 ± 0.17 [6]	1.67 ± 0.20* [6]	0.94 ± 0.12 [6]	1.31 ± 0.15 [6]	Arambula et al., 2018
		***Esr2* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	M	–	1 ± 0.23 [6]	0.91 ± 0.27 [5]	2.38 ± 0.39** [7]	1.42 ± 0.17 [7]	0.84 ± 0.15 [5]	1.34 ± 0.42 [6]	Arambula et al., 2018
		***Grm5* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	F	–	1 ± 0.049 [4]	1.29 ± 0.097 [5]	1.62 ± 0.27 [6]	1.89 ± 0.28** [6]	1.71 ± 0.31* [5]	2.01 ± 0.24* [6]	Arambula et al., 2018
		***Grm5* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	M	–	1 ± 0.14 [6]	0.81 ± 0.20 [5]	1.54 ± 0.097** [7]	1.38 ± 0.17 [7]	0.76 ± 0.097 [5]	1.36 ± 0.15 [6]	Arambula et al., 2018
		***Oxtr* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	F	–	1 ± 0.15 [7]	1.37 ± 0.13* [6]	1.90 ± 0.35** [6]	1.97 ± 0.34* [6]	1.12 ± 0.20 [6]	1.58 ± 0.15** [6]	Arambula et al., 2018
		***Oxtr* expression (amygdala)[3]** mean ± SE (relative abundance) [n]	M	–	1 ± 0.12 [6]	0.73 ± 0.15 [5]	1.95 ± 0.21** [7]	1.37 ± 0.11* [7]	0.68 ± 0.13 [5]	1.07 ± 0.16 [6]	Arambula et al., 2018
		***Esr1* expression (hypothalamus)[4]** mean (relative abundance) [n]	F	–	1 [5]	6.06** [6]	3.30 [5–6]	5.25* [5]	1.48 [5–6]	3.51** [5]	Arambula et al., 2016
		***Esr2* expression (hypothalamus)[4]** mean (relative abundance) [n]	F	–	1 [5]	5.51** [6]	2.59 [5–6]	3.53* [5]	1.57 [5–6]	2.81* [5]	Arambula et al., 2016
		***Oxt* expression (hypothalamus)[4]** mean (relative abundance) [n]	F	–	1 [5]	8.80* [6]	2.72 [5–6]	6.20* [5]	1.31 [5–6]	7.54 [5–6]	Arambula et al., 2016
		***Oxt* expression (hypothalamus)[4]** mean (relative abundance) [n]	M	–	1 [5]	0.89 [5–6]	20.8* [5]	4.21 [5–6]	1.28 [5–6]	3.42 [5–6]	Arambula et al., 2016
		***Slc32a1* expression (hypothalamus)[4]** mean (relative abundance) [n]	F	–	1 [6]	5.25** [6]	3.34 [5–6]	4.40** [5]	1.43 [5–6]	3.76* [5]	Arambula et al., 2016

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
		***Esr2* expression (hippocampus)[4]** mean (relative abundance) [n]	M	–	1 [4]	1.08 [5–6]	1.98 [5–6]	3.03 [5–6]	1.23 [5–6]	9.45* [5]	Arambula et al., 2016
		***Oxt* expression (hippocampus)[4]** mean (relative abundance) [n]	F	–	1 [5]	2.04 [5–6]	3.73* [5]	2.63 [5–6]	1.52 [5–6]	1.47 [5–6]	Arambula et al., 2016
		***Oxt* expression (hippocampus)[4]** mean (relative abundance) [n]	M	–	1 [5]	0.65 [5–6]	0.53 [5–6]	0.94 [5–6]	0.44* [6]	0.43* [4]	Arambula et al., 2016
SD, PND 28	**Morphology**	**Right medial amygdala (MePD) volume[5]** mean (µg/m^3) (lower 95% CI, upper 95% CI) [n]	F	–	7.91E+07 ± 5.52E+06 [10]	8.98E+07 ± 4.69E+06 [10]	8.93E+07 ± 4.57E+06 [9]	–	1.05E+08 ± 3.17E+06** [10]	–	Arambula et al., 2017
SD, PND 28	**Hormone Function**	***Oxtr* binding (hypothalamus BNSTld)[6]** mean ± SE [n]	M	–	0.32 ± 0.028 [8]	0.47 ± 0.016** [9]	0.44 ± 0.033** [9]	–	0.37 ± 0.019 [9]	–	Witchey et al., 2019
SD, PND 97 or 111	**Behavior**	**Barnes maze, frequency of sniffing incorrect hole[7]** mean ± SE [n]	M + F	–	5.55 ± 1.4 [12]	4.0 ± 1.0 [12]	6.5 ± 1.3 [12]	NA [12]	10.8 ± 2.2* [12]	NA [12]	Johnson et al., 2016
SD, PND 101 or 115	**DNA Methylation[8]**	***Dnmt3b* methylation (hypothalamus)** mean ± SE [n]	M	–	58.4 ± 5.4 [5]	–	–	–	50.8 ± 5.3*** [5]	–	Cheong et al., 2018
	Gene Expression[8]	***Dnmt3b* expression (hypothalamus)** mean (fold change) (lower 95% CI, upper 95% CI) [n]	M	–	1 [5]	–	–	–	0.08 (0.02, 0.27)*** [5]	–	Cheong et al., 2018
		***Bdnf* expression (hypothalamus)** mean (fold change) (lower 95% CI, upper 95% CI) [n]	M	–	1 [5]	–	–	–	0.09 (0.02, 0.33)*** [5]	–	Cheong et al., 2018
		***Esr1* expression (hypothalamus)** mean (fold change) (lower 95% CI, upper 95% CI) [n]	M	–	1 [5]	–	–	–	0.16 (0.03, 0.83)* [5]	–	Cheong et al., 2018
		***Oxtr* expression (hypothalamus)** mean (fold change) (lower 95% CI, upper 95% CI) [n]	M	–	1 [5]	–	–	–	0.20 (0.06, 0.68)* [5]	–	Cheong et al., 2018
		***Oxtr* expression (hippocampus)** mean (fold change) (lower 95% CI, upper 95% CI) [n]	M	–	1 [5]	–	–	–	11.8 (1.08, 129)[10] [5]	–	Cheong et al., 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
SD, PND 101–107 or 115–119	**DNA Methylation[8]**	***Bdnf* methylation (hippocampus)** mean ± SE [n]	F	–	35.3 ± 19.8 [5]	–	–	–	63.3 ± 16.8*** [5]	–	Cheong et al., 2018
SD, PND 101–107 or 115–119	**Gene Expression[8]**	***Avp* expression (hippocampus)** mean (fold change) (lower 95% CI, upper 95% CI) [n]	F	–	1 [5]	–	–	–	6.05 (1.15, 31.8)* [5]	–	Cheong et al., 2018
CD, 2 yr	**Pathology[9]**	**Compression (brain stem)** incidence[11]/n, % incidence (severity profile)	F	NS	14/50, 28% (1|3|4|6)	13/48, 27% (3|4|2|4)	7/46, 15%N^ (5|0|2|0)	14/49, 29% (2|5|3|4)	13/50, 26% (2|3|5|3)	17/46, 37% (2|5|5|5)	NTP, 2018
SD, 2 yr	**Pathology[9]**	**Ventricle dilatation (cerebrum)** incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	M	↓#,^,*	7/49, 14% 7/38.9, 18% (4|3|0|0)	7/48, 15% 7/36.0, 19% (4|2|1|0)	6/48, 13% 6/36.7, 16% (3|3|0|0)	4/50, 8% 4/32.1, 12% (3|1|0|0)	3/50, 6% 3/37.0, 8% (1|1|1|0)	2/46, 4% 2/28.2, 7% (0|2|0|0)	NTP, 2018

CD = continuous dose; SD = stop dose; hyphen (–) = not evaluated; NA = not available; MePD = posterodorsal subnucleus of the medial amygdala.
[1]Shading indicates statistical significance. Statistical tests varied by reference (see subsequent footnotes).
[2]↑ = positive trend, ↓ = negative trend, NS = no significant trend.
[3]Statistical analyses by Mann-Whitney U test (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).
[4]Statistical analyses were conducted by the Mann-Whitney U test (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).
[5]Statistical analyses were conducted by ANOVA followed by Dunnett's multiple comparison post hoc test (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).
[6]Statistical analyses by one-way ANOVA followed by Fisher's exact LSD post hoc test (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).
[7]Statistical analyses by Fisher's LSD (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).
[8]Statistical analyses were conducted by ANOVA followed by Tukey's multiple comparison test (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$; ***, statistically significant at $p \leq 0.001$).
[9]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected.
Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.
Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.
Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests, which are conducted on simple incidence data. All pairwise tests were one-sided and not corrected for multiple comparisons.
Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs.
Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; N superscript, negative trend, or negative relative to control.
[10]Borderline significance at p value of 0.06.
[11]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.

Chapter 2. Cardiac

Findings of the CLARITY-BPA core study (NTP 2018) and the investigational study by the laboratory of Scott Belcher (Gear et al. 2017) with respect to the potential effects of BPA on the heart are summarized in this chapter. All individual animal data for these studies are available online (https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA). The study details, including endpoints measured and timing of assessment, are detailed in Table 4. The statistically significant findings from the investigational study and the statistically significant findings from the core study on related endpoints are included in Table 5.

Table 4. Study Details and Summary of Measured Cardiac Endpoints

Endpoint Category	Specific Measure	Gear et al. (2017)[1]						NTP (2018)[2]			
		Age at Assessment									
		21 d		90 d		6 mo		1 yr		2 yr	
Biochemical[3]	Cardiac biomarker	–	–	–	–	–	–	●	□	–	–
Organ Weight[4]	Heart weight	●	–	●	□	●	□	●	□	–	–
Pathology[5]	Heart	●	–	●	□	●	□	●	□	●	□
Morphology[6]	Heart	●	–	●	□	●	□	–	–	–	–

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.
yr = year; d = day; mo = month; bw = body weight; PND = postnatal day.
[1]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at PND 21, PND 90, or PND 180 (6 mo) and examined for effects. Animals were sacrificed also at 1 yr, but the biological samples collected were not analyzed.
[2]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr or 2 yr and examined for effects.
[3]NTP 2018 endpoint list: troponin T, troponin I.
[4]NTP 2018 endpoint list: heart weight: absolute, relative to brain, relative to bodyweight.
Gear et al. 2017 endpoint list: heart weight: absolute; heart weight: relative to bodyweight.
[5]NTP 2018 endpoint list: comprehensive assessment of neoplastic and nonneoplastic lesions, including cardiomyopathy.
Gear et al. 2017 endpoint list: cardiomyopathy; diffuse degeneration; fibrosis: collagen, focal fibrosis; inflammation/necrosis.
[6]Gear et al. 2017 endpoint list: left ventricular wall thickness.

Study Summaries

Core Study: NTP (2018)

Methods

The endpoints evaluated in the core study related to the heart included heart weights and serum troponin assays at 1 year and histopathology at 1 year and study termination (Table 4).

Findings

In males, detectable levels of serum troponin T occurred with a positive trend at 1 year in the BPA-exposed continuous-dose arm (Table 5). There was a greater incidence of cardiomyopathy at 1 year in the 2.5 and 25 µg BPA/kg bw/day stop-dose groups of males compared to controls (RTE test; see Introduction for explanation of statistical tests used). There was a significant positive trend in cardiomyopathy incidence in stop-dose males at 2 years (Poly-3). At 2 years, mineralization in the heart was lower than in controls in the 2.5 µg BPA/kg bw/day dose group

of stop-dose males (Poly-3, JT/SW, and RTE) and in the 250 µg BPA/kg bw/day stop-dose group of males (JT/SW and RTE).

In females, there was an increase in cardiomyopathy in the stop-dose arm at 2.5, 250, 2,500, and 25,000 µg BPA/kg bw/day versus the control at 2 years (JT/SW and RTE). Females in the 25 µg BPA/kg bw/day continuous-dose arm had decreased cardiomyopathy relative to controls at 2 years (RTE).

Ethinyl estradiol

Cardiomyopathy was greater than in controls in the high-dose EE2 continuous-dosed female group at 1 year (CAFE, JT/SW, and RTE) and 2 years (JT/SW and RTE).

Author conclusions

The authors stated that the high background incidence of this lesion at terminal sacrifice and variability in incidence and severity scores across groups were "…within the confines of background variation…" (Camacho et al. 2019) and "…make the toxicological significance of these results questionable…" (NTP 2018).

Investigational Study: Gear et al. (2017)

Methods

Animals were taken from the continuous- and stop-dose arms at PND 21, PND 90, or 6 months of age. Hearts were fixed in 10% formalin and shipped to the Belcher lab, where they were processed for histopathological evaluation, morphometric evaluation of ventricular wall thickness, and collagen content (Table 4).

Findings

Heart weights were lower in stop-dose females exposed to 2.5 µg BPA/kg bw/day at 6 months. No effects on left ventricular wall thickness were noted at PND 21, but at 90 days there was a decrease in wall thickness in the female stop-dose 2.5 µg BPA/kg bw/day group. There was no evidence of an increase in fibrosis (collagen staining) in any group by morphometry, and decreased collagen accumulation in BPA-exposed females at 90 days and 6 months was described as "minor." Cardiomyopathy was a common lesion seen in the study with 9/10 control males and 6/10 control females reported as showing evidence of this lesion as early as the 21-day time point. The authors reported increases in incidence and severity of cardiomyopathy in females at 2.5 and 250 µg BPA/kg bw/day as indicated by large effect size (although effects were not statistically significant), and an increased incidence and severity ($p < 0.05$) of cardiomyopathy in BPA-exposed females at 25,000 µg/kg bw/day at 21 days. The authors reported occurrences of "diffuse degeneration phenotype" in some BPA and EE2 dose groups, but not in controls at PND 21 and PND 90. Diffuse degeneration was reported to involve much of the myocardium. Increases in cardiomyopathy were no longer evident with BPA exposures at 6 months. Many of the animals from all dosed and control groups evaluated for cardiac effects at age 6 months were housed briefly with animals receiving 250,000 µg BPA/kg bw/day and may have experienced some BPA contamination as a result (see Introduction for further details).

Ethinyl estradiol

In continuous-dose females exposed to EE2, absolute heart weights were increased at PND 90 at 0.5 and 0.05 μg/kg bw/day and at 6 months at 0.5 μg/kg bw/day. Heart weights relative to body weight were increased at 0.5 μg EE2/kg bw/day in both continuous-dose and stop-dose groups at PND 90 and in continuous-dose females only at age 6 months. At PND 21, fibrosis was increased in males at 0.5 μg EE2/kg bw/day, and progressive cardiomyopathy was greater than in controls in both EE2 treatments groups in continuous-dose females. Fibrosis was decreased in 0.5 μg EE2/kg bw/day stop-dose females at 6 months.

Author conclusions

The authors concluded that "exposures to either BPA or EE[2] increased incidence and severity of progressive cardiomyopathy in females at PND 21 and increased the severity of cardiomyopathy in both sexes at PND 90" (Gear et al. 2017).

Table 5. Summary of Statistically Significant Cardiac Endpoints

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
CD, 21 d	**Pathology**	**Cardiomyopathy**[3,4] incidence[5]/n, % incidence (severity score)	F	–	6/10, 60% (0.8)	10/10, 100% (1.8)	9/10, 90% (1.4)	10/10, 100% (1.8)	7/8, 88% (1.1)	10/10, 100%* (1.9)	Gear et al., 2017
SD, 90 d	**Morphology**	**Left ventricular wall thickness**[3] mean ± SD, (mm) [n]	F	–	2.60 ± 0.29 [10]	2.30 ± 0.22* [8]	2.49 ± 0.20 [10]	2.47 ± 0.28 [10]	2.49 ± 0.20 [8]	2.49 ± 0.22 [10]	Gear et al., 2017
SD, 6 mo	**Organ Weight**	**Heart weight: absolute**[3] mean ± SD, (mg) [n]	F	–	1,144 ± 136 [10]	1,011 ± 47.0* [10]	1,129 ± 63.8 [10]	1,067 ± 102 [10]	1,111 ± 81.4 [10]	1,085 ± 133 [10]	Gear et al., 2017
CD, 1 yr	**Biochemical**	**Troponin T**[6] detects/total, % detects	M	↑**	11/18, 61%	10/22, 46%	11/18, 61%	14/24, 58%	12/18, 67%	17/20, 85%	NTP, 2018
SD, 1 yr	**Pathology**[7]	**Cardiomyopathy** incidence/n, % incidence (severity profile)	M	NS	17/20, 85% (12|3|2|0)	20/20, 100%^ (10|7|2|1)	19/20, 95%^^ (7|7|3|2)	13/19, 68% (10|3|0|0)	18/20, 90% (14|3|1|0)	19/22, 86% (9|8|2|0)	NTP, 2018
CD, 2 yr	**Pathology**[7]	**Cardiomyopathy** incidence/n, % incidence (severity profile)	F	NS	35/50, 70% (24|10|1|0)	30/48, 62% (18|7|4|1)	24/46, 52%N^ (18|5|1|0)	35/49, 71% (25|9|1|0)	33/50, 66% (24|7|1|1)	33/46, 72% (23|9|0|1)	NTP, 2018
SD, 2 yr	**Pathology**[7]	**Cardiomyopathy** incidence/n, % incidence (severity profile)	F	↑##,^^	32/50, 64% (26|3|3|0)	37/50, 74%#9,^ (22|13|2|0)	38/48, 79% (29|7|2|0)	37/50, 74%#,^ (21|14|2|0)	35/50, 70%#,^ (17|13|5|0)	35/46, 76%##,^^ (16|14|5|0)[8]	NTP, 2018
		Cardiomyopathy Poly-3 incidence/ adjusted n, % incidence (severity profile)	M	↑*	45/48.1, 94% (8|20|11|6)	44/45.7, 96% (13|18|7|6)	45/46, 98% (10|16|12|7)	48/48.9, 98% (15|21|10|2)	47/47.4, 99% (12|13|12|10)	41/41.7, 98% (9|16|10|6)	NTP, 2018
		Blood vessel mineralization incidence/n, % incidence (severity profile)	M	NS	8/50, 16% (0|0|1|7)	2/48, 4%N#9,^ (0|0|0|2)	6/48, 13% (0|1|2|3)	4/50, 8% (1|1|0|2)	7/50, 14% (0|1|1|5)	4/46, 9% (1|0|0|3)	NTP, 2018
		Heart mineralization incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	M	NS	9/50, 18% 9/39.6, 23% (0|1|3|5)	1/48, 2%N##9,^^ 1/34.1, 3%N* (0|0|0|1)	6/48, 13% 6/37.3, 16% (0|1|3|2)	2/50, 4%N##9,^^ 2/32.1, 6% (0|1|0|1)	7/50, 14% 7/38.3, 18% (0|1|1|5)	4/46, 9% 4/28.7, 14% (0|1|1|2)	NTP, 2018

CD = continuous dose; SD = stop dose; d = day; mo = month; yr = year.

[1]Shading indicates statistical significance. Statistical tests varied by reference (see subsequent footnotes).
[2]↑ = positive trend; ↓ = negative trend; NS = no significant trend; hyphen (–) = not evaluated.
[3]Data analysis was performed using Dunnett's multiple comparison tests. For pathology severity scores, a rank order ANOVA Kruskal-Wallis H test with Dunn's multiple comparisons tests were used (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).
[4]Percent incidence was calculated from study data.
[5]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.
[6]A generalized linear logistic regression model was used to model troponin T detection level. Troponin T means and medians were not analyzed because of the relatively high number of nondetects, which were assigned a value of half the limit of detection. Detects/total refers to the number of samples above the limit of detection over the total number of samples. One sample was taken per animal.
[7]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected.
Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.
Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.
Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests. All pairwise tests were one-sided and not corrected for multiple comparisons.
Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs.
Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; N superscript, negative trend, or negative relative to control.
[8]In the sensitivity analysis that excluded all animals that overlapped with animals treated with 250,000 µg BPA/kg bw/day, there was a significant Poly-3 test ($p = 0.049$) for the comparison of the 25,000 µg BPA/kg bw/day dose group to the vehicle controls (Poly-3 incidences 27/30.8 [88%] versus 22/31.6 [70%]).
[9]SW test was nonsignificant due to the assumption of monotonicity; thus, this JT/SW data analysis result was not summarized in the CLARITY-BPA integrated report.

Chapter 3. Immune

Findings of the CLARITY-BPA core study (NTP 2018) and the investigational study by the laboratory of Norbert Kaminski (Li et al. 2018a; 2018b) with respect to the potential effects of BPA on immune endpoints are summarized below. All individual animal data for these studies are available online (https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA). The study details, including endpoints measured and timing of assessment, are detailed in Table 6. The statistically significant findings from each investigational study and the statistically significant findings from the core study on related endpoints are included in Table 7 (NTP 2018), Table 8 (Li et al. 2018b), and Table 9 (Li et al. 2018a).

Study Summaries

Core Study: NTP (2018)

Methods

The immune-related endpoints evaluated in the core study were limited to observational measures rather than assessment of immune function. Endpoints included spleen and thymus weights at interim sacrifice (1 year), peripheral blood leukocyte phenotyping (e.g., counts and percentage of neutrophils, eosinophils) at 1 year, and histopathology of spleen, thymus, and bone marrow at 1 year and study termination (2 years).

Findings

Leukocyte measures

Several changes in leukocyte populations were reported in BPA-exposed animals (Table 7). The percentage of neutrophils was decreased in males with a negative trend and the percentage of basophils was increased in females with a positive trend in BPA stop-dose animals at 1 year. The number of monocytes was increased with a positive trend in BPA continuous-dose females at 1 year. Eosinophils were decreased in both the males and females in the 250 µg BPA/kg bw/day continuous-dose group at 1 year (number in females and percentage in males).

Thymus

Changes in thymic atrophy were reported in some BPA-exposed animals, but the direction of the effect was not consistent across doses or treatment arms. In females, there was increased incidence and/or severity of thymic atrophy in several dose groups (e.g., greater severity in stop-dose animals at 2 years for the 25 and 2,500 µg BPA/kg bw/day groups [RTE]). In males, there was a trend for decreased thymic atrophy in stop-dose BPA-exposed males at 2 years and decreased incidence at 25,000 µg BPA/kg bw/day (JT/SW and RTE).

Spleen

There was a negative trend for spleen pigmentation in stop-dose BPA females at 1 year (CAFE). There was a positive trend for greater hematopoietic cell proliferation in the spleen in continuous-dose BPA-exposed males at 1 year (Poly-3, JT/SW, and RTE) and by pairwise comparison of the 25,000 µg BPA/kg bw/day group with controls (JT/SW and RTE). There were also significant increases in pigmentation in the spleen for stop-dose males at 1 year by trend analysis for BPA treatment and by pairwise comparison in the 250 µg BPA/kg bw/day group

compared to controls (CAFE). There was increased lymphoid hyperplasia in the spleen for the 250 and 2,500 µg BPA/kg bw/day stop-dose groups (Poly-3 and RTE) and a positive trend for increased lymphoid hyperplasia in the spleen in stop-dose BPA-exposed males at 2 years (Poly-3).

Bone marrow

In the stop-dose BPA-exposed males, there were significant increases in myeloid cell hyperplasia in the bone marrow of the 25 µg BPA/kg bw/day group after 1 year (RTE). There were also higher rates of bone marrow hypocellularity in stop-dose males at 2 years in the 250 µg BPA/kg bw/day group (Poly-3 and RTE) and in the 25,000 µg BPA/kg bw/day group (Poly-3, JT/SW, and RTE). There was an increased incidence of bone marrow hypocellularity in continuous-dose BPA-exposed females in the 25,000 µg BPA/kg bw/day group at 2 years (JT/SW and RTE). In contrast, for continuous-dose males, there was a decrease in hypocellularity in the bone marrow for the 250 and 2,500 µg BPA/kg bw/day groups (Poly-3 and RTE) and a negative trend for BPA at 2 years (Poly-3, JT/SW, and RTE).

There was an increasing trend for incidence of malignant lymphoma in bone marrow and spleen in BPA-exposed stop-dose males at age 2 years (Poly-3). The incidence of malignant lymphoma was also significantly higher in kidney, liver, and dorsolateral prostate of stop-dose BPA-exposed males at 2 years by trend analysis (Poly-3) as well as by pairwise comparison of 25,000 µg BPA/kg bw/day versus controls for dorsolateral prostate (Poly-3). The malignant lymphomas in these five organs were diagnosed in the same animals indicating a systemic condition. In contrast, a significant negative trend for the incidence of malignant lymphoma was observed in both bone marrow and spleen in BPA-exposed continuous-dose males at age 1 year (CAFE).

Ethinyl estradiol

The number and percentage of eosinophils was decreased in continuous-dosed EE2 females in the high-dose group at age 1 year (0.5 µg EE2/kg bw/day). Bone marrow hyperplasia was increased in continuous-dosed EE2 females in the high-dose group (0.5 µg EE2/kg bw/day) (JT/SW and RTE), and there was a positive trend for EE2 dosed females at 2 years (Poly-3, JT/SW and RTE). Spleen pigmentation was also increased in continuous-dosed EE2 females in the high-dose group at 1 year (JT/SW and RTE) and 2 years (Poly-3, JT/SW and RTE), and there was a positive trend for EE2 dose females at 1 year (JT/SW and RTE) and 2 years (Poly-3, JT/SW and RTE). In continuous-dosed EE2 males, bone marrow hypocellularity was decreased in the low-dose group at 2 years (0.05 µg EE2/kg bw/day) (RTE).

Author conclusions

The authors reported significant dose trends in stop-dose BPA-exposed males for malignant lymphoma in the spleen and bone marrow and several other organs (liver, dorsolateral lobe prostate, and kidney). They noted that incidence of malignant lymphoma never exceeded 16.8% and there were no significant differences for animals continuously dosed with BPA. The authors also stated that several significantly greater incidences of nonneoplastic lesions were observed for various BPA dose groups compared to controls for both females and males; however, none were repeated across treatment arms or consistently found across dose groups.

Table 6. Study Details and Summary of Measured Immune Endpoints

Endpoint Category	Specific Measure	Li et al. (2018b)[2]		Li et al. (2018a)[3]	Li et al. (2018b)[2]	Li et al. (2018a)[3]	NTP (2018)[1]		Li et al. (2018b)[2]	Li et al. (2018a)[3]	NTP (2018)[1]	
		Age at Assessment										
		PND 21	PND 90		6 mo		1 yr				2 yr	
Leukocyte Measures[4]	Peripheral blood leukocyte count, differential	–	–	–	–	–	●	□	–	–	–	–
	Spleen leukocyte count, differential	●	●	–	●	–	–	–	●	–	–	–
	Thymus leukocyte count, differential	●	–	–	–	–	–	–	–	–	–	–
Cellularity[5]	Spleen	●	●	–	●	–	–	–	●	–	–	–
	Thymus	●	–	–	–	–	–	–	–	–	–	–
Immune Function[6]	Antibody response	–	–	●	–	●	–	–	–	●	–	–
	Lymphoproliferation (PWM, LPS, etc.)	–	–	●	–	●	–	–	–	●	–	–
	Leukocyte activation (NK, T cell, etc.)	–	–	●	–	●	–	–	–	●	–	–
Organ Weight[7]	Spleen weight	–	–	–	–	–	●	□	–	–	–	–
	Thymus weight	–	–	–	–	–	●	□	–	–	–	–
Pathology[8]	Spleen	–	–	–	–	–	●	□	–	–	●	□
	Thymus	–	–	–	–	–	●	□	–	–	●	□
	Bone marrow	–	–	–	–	–	●	□	–	–	●	□

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.

yr = year; d = day; PND = postnatal day; mo = month; bw = body weight; lipopolysaccharide (LPS); pokeweed mitogen (PWM); natural killer (NK); continuous dose (CD)

[1]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 year or 2 years and examined for effects.

[2]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose BPA treatment. Animals were sacrificed at PND 21, PND 90, PND 180 (6 mo), and 1 yr and examined for effects.

[3]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose BPA treatment. Animals were sacrificed at PND 90, PND 180 (6 mo), and 1 yr and examined for effects.

[4]NTP 2018 endpoint list: white blood cell count; basophils: count, %; eosinophils: count, %; lymphocytes: count, %; monocytes: count, %; neutrophils: count, %.
Li et al. 2018a endpoint list: % antigen presenting cells; % B cells; % CD3+ T cells; % CD4+ T cells; % CD8+ T cells; % classic dendritic cells; % macrophages; % mature classic dendritic cells; % monocytes/macrophages/granulocytes; % NK cells; % NKT cells [spleen, all time points]. % CD3+ T cells; % CD4+ T cells; % CD4+CD8+ T cells; % CD8+ T cells [thymus, PND 21].

[5]Li et al. 2018a endpoint list: spleen cellularity [all time points]; thymus cellularity [PND 21].

[6]Li et al. 2018b endpoint list: intracellular IgM (LPS or PWM activation); secreted IgM (LPS or PWM activation); splenocyte proliferation (LPS, PWM or anti-CD3/CD28 activation); % CD86+ macrophages/dendritic cells (24 hr after LPS, 48 hr after LPS); % MCHII+ macrophages/dendritic cells (24 hr after LPS, 48 hr after LPS); % CD86+ monocytes/macrophages/granulocytes (24 hr after LPS, 48 hr after LPS); % MCHII+ monocytes/macrophages/granulocytes (24 hr after LPS, 48 hr after LPS); % CD25+ T cells (anti-CD3CD28 stimulation); MFI of CD25 in T cells (anti-CD3CD28 stimulation) [all time points].
% CD80+ NK cells (24 hr after LPS); % CD80+ NK cells (48 hr after LPS); % CD86+ NK cells (24 hr after LPS); % CD86+ NK cells (48 hr after LPS) [6 mo and 1 yr].

[7]NTP 2018 endpoint list: spleen weight: absolute, relative to brain, relative to body weight; thymus weight: absolute, relative to brain, relative to body weight.

[8]NTP 2018 endpoint list: comprehensive assessment of neoplastic and nonneoplastic lesions (e.g., malignant lymphoma of bone marrow and spleen, thymus atrophy).

Table 7. Summary of Statistically Significant Immune Endpoints in NTP (2018)

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]						
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000
CD, 1 yr	Leukocyte Measures[3]	**Eosinophils: count** mean ± SE, (10^3/mm^3) [n]	F	NS	0.12 ± 0.01 [21]	0.09 ± 0.01 [22]	0.11 ± 0.01 [21]	0.09 ± 0.01* [22]	0.10 ± 0.01 [20]	0.10 ± 0.01 [24]
		Eosinophils: % mean ± SE, [n]	M	NS	1.71 ± 0.14 [18]	1.74 ± 0.23 [22]	1.53 ± 0.13 [18]	1.23 ± 0.1* [24]	1.29 ± 0.09 [18]	1.44 ± 0.16 [21]
		Monocytes: count mean ± SE, (10^3/mm^3) [n]	F	↑*	0.6 ± 0.1 [21]	0.6 ± 0.1 [22]	0.8 ± 0.1 [21]	0.7 ± 0.1 [22]	0.7 ± 0.1 [20]	0.6 ± 0.1 [24]
	Pathology[4]	**Spleen, cell proliferation** incidence[5]/n, % incidence (severity profile)	M	↑*,#,^	0/22, 0% (–)	0/22, 0% (–)	0/20, 0% (–)	0/24, 0% (–)	0/18, 0% (–)	2/22, 9%##,^ (0|2|0|0)
		Spleen, malignant lymphoma incidence/n, % incidence	M	↓*	2/22, 9%	0/22, 0%	0/20, 0%	0/24, 0%	0/18, 0%	0/22, 0%
		Bone marrow, malignant lymphoma incidence/n, % incidence	M	↓*	2/22, 9%	0/22, 0%	0/20, 0%	0/24, 0%	0/18, 0%	0/22, 0%
		Thymus, atrophy incidence/n, % incidence (severity profile)	F	NS	20/23, 87% (0|2|8|10)	20/22, 91% (1|4|7|8)	18/22, 82%N#6 (0|6|8|4)N^	21/24, 88% (2|3|7|9)	16/20, 80% (0|4|5|7)	22/24, 92% (0|5|10|7)
SD, 1 yr	Leukocyte Measures[3]	**Neutrophils: %** mean ± SE, [n]	M	↓*	23.9 ± 2.0 [19]	28.4 ± 2.3 [20]	25.9 ± 2.2 [19]	25.2 ± 2.1 [19]	21.0 ± 0.8 [20]	21.8 ± 1.6 [22]
		Basophils: % mean ± SE, [n]	F	↑*	0.1 ± 0 [20]	0.2 ± 0 [22]	0.2 ± 0 [20]	0.3 ± 0.1 [22]	0.1 ± 0 [20]	0.1 ± 0 [19]
	Pathology[4]	**Spleen, pigmentation** incidence/n, % incidence (severity profile)	M	↑*	12/20, 60% (4|6|2|0)	14/20, 70% (5|9|0|0)	13/20, 65% (7|5|1|0)	18/19, 95%* (11|7|0|0)	15/20, 75% (5|10|0|0)	18/22, 82% (6|11|0|1)
		Bone marrow, cell hyperplasia incidence/n, % incidence (severity profile)	M	NS	0/20, 0% (–)	0/20, 0% (–)	2/20, 10%#6,^ (0|0|1|1)	0/19, 0% (–)	1/20, 5% (0|0|1|0)	0/22, 0% (–)
		Spleen, pigmentation incidence/n, % incidence (severity profile)	F	↓*	20/20, 100% (6|6|5|3)	22/22, 100% (2|7|10|3)	20/20, 100% (5|8|3|4)	22/22, 100% (5|7|9|1)	20/20, 100% (0|9|10|1)	20/22, 91% (1|6|10|3)

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]						
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000
		Thymus, atrophy incidence/n, % incidence (severity profile)	F	NS	18/20, 90% (0|8|5|5)	19/22, 86% (0|8|2|9)	18/20, 90% (0|2|4|12)^	17/22, 77% (0|5|6|6)	18/20, 90% (0|5|5|8)	16/22, 73% (0|4|4|8)
CD, 2 yr	**Pathology[4]**	**Bone marrow, hypocellularity** incidence/n, % incidence (severity profile)	F	NS	2/50, 4% (0|0|1|1)	3/48, 6% (0|0|1|2)	1/46, 2% (0|0|0|1)	2/49, 4% (0|0|2|0)	2/49, 4% (0|0|1|1)	6/46, 13%#,^ (0|0|5|1)
		Bone marrow, hypocellularity incidence/n, % incidence Poly-3 incidence/adjusted n, % incidence (severity profile)	M	↓*,#,^	5/48, 10% 5/33.3, 15% (0|0|5|0)	7/48, 15% 7/35.1, 20% (0|0|4|3)	5/48, 10% 5/33.3, 15% (0|0|4|1)	0/49, 0%N#6,^ 0/32.9, 0%N* (–)	0/50, 0%N#6,^ 0/33.6, 0%N* (–)	4/45, 9% 4/29.0, 14% (0|0|2|2)
		Thymus, atrophy Poly-3 incidence/adjusted n, % incidence (severity profile)	F	↑*	47/49.3, 95% (0|2|4|41)	47/47, 100% (0|2|6|39)	43/43.2, 99.5% (0|1|7|35)	47/47.2, 99.6% (0|0|4|43)	47/47.1, 99.8% (0|3|8|36)	44/44, 100% (0|0|4|40)
SD, 2 yr	**Pathology[4]**	**Bone marrow, hypocellularity** incidence/n, % incidence Poly-3 incidence/adjusted n, % incidence (severity profile)	M	NS	0/47, 0% 0/35.5, 0% (–)	3/47, 6% #6 3/33.7, 9% (0|0|1|2)	2/46, 4% 2/34.8, 6% (0|0|1|1)	4/50, 8%#6,^ 4/31.8, 13%* (0|0|4|0)	2/49, 4% 2/36.1, 6% (0|0|2|0)	4/45, 9%#,^ 4/28.7, 14%* (0|0|4|0)
		Bone marrow, malignant lymphoma Poly-3 incidence/adjusted n, % incidence	M	↑**	1/36.1, 3%	0/32.6, 0%	0/34.3, 0%	3/32.9, 9%	2/37.4, 5%	5/29.7, 17%
		Spleen, lymphoid hyperplasia incidence/n, % incidence Poly-3 incidence/adjusted n, % incidence (severity profile)	M	↑*	1/47, 2% 1/35.7, 3% (0|1|0|0)	2/47, 4% 2/33.1, 6% (0|0|1|1)	3/47, 6% 3/36.3, 8% (0|3|0|0)	6/49, 12%#6,^ 6/32.5, 18%* (0|5|1|0)	6/49, 12%#6,^ 6/37.5, 16% (0|6|0|0)	3/45, 7% 3/27.9, 11% (0|3|0|0)
		Spleen, malignant lymphoma Poly-3 incidence/adjusted n, % incidence	M	↑**	1/36.1, 3%	0/32.6, 0%	1/35.4, 3%	3/32.5, 9%	2/37.4, 5%	5/29.7, 17%
		Thymus, atrophy incidence/n, % incidence (severity profile)	F	NS	48/49, 98% (0|2|9|37)	48/50, 96% (0|1|7|40)	47/48, 98%#6 (0|0|4|43)^	47/50, 94% (1|0|6|40)	47/49, 96%#6 (0|0|3|44)^	42/46, 91% (0|0|4|38)

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]						
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000
		Thymus, atrophy incidence/n, % incidence	M	↓#,^^	47/48, 98% (0|0|1|46)	45/48, 94% (0|0|1|44)	44/46, 96% (0|0|0|44)	43/47, 92% (0|1|1|41)	46/48, 96% (0|0|1|45)	38/42, 91%N##,^^ (0|0|5|33)

CD = continuous dose; SD = stop dose; d = day; mo = month; yr = year; bw = body weight.
[1]Shading indicates statistical significance.
[2]↑ = positive trend, ↓ = negative trend, NS = no significant trend, hyphen (–) = not evaluated.
[3]Non-parametric ANOVA based on mid-ranks was used to evaluate the effect of treatment and Dunnett's adjustment was used for pairwise multiple comparisons relative to the control; orthogonal contrasts were used to test for trend (*, statistically significant at $p \leq 0.05$). All statistical tests are two-sided.
[4]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected.
Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.
Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.
Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests, which are conducted on simple incidence data. All pairwise tests were one-sided and not corrected for multiple comparisons.
Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs.
Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; N superscript, negative relative to control.
[5]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.
[6]SW test was nonsignificant due to the assumption of monotonicity; thus, this JT/SW data analysis result was not summarized in the CLARITY-BPA integrated report.

Investigational Study: Li et al. (2018b)

Methods

Animals taken from the continuous-dose arms were euthanized on PND 21, PND 90, 6 months, or 1 year of age. Thymus (PND 21) and spleens (PND 21, PND 90, 6 months, and 1 year) were collected in medium on ice and shipped to the Kaminski laboratory for processing the following day. Thymocytes and splenocytes were isolated, washed, counted, stained for cell surface markers using specific antibodies, and examined with a flow cytometer to identify lymphocyte and myeloid cell populations.

Findings

The authors reported altered composition of several splenic lymphocyte or myeloid cell populations in BPA-exposed rats; however, the results were usually specific to only one sex, age, or BPA dose group (Table 8). Only two results were identified in more than one dose group; a decrease in the percentage of splenic antigen presenting cells in males at 1 year in the 2.5 and 25 µg BPA/kg bw/day dose groups and a decrease in the percentage of mature splenic dendritic cells in males at 6 months in the 2.5 and 2,500 µg BPA/kg bw/day dose groups.

Ethinyl estradiol

There were changes is several lymphocyte and myeloid cell populations from the thymus and spleen in rats exposed to EE2. Most changes were reported at the high dose (0.5 µg EE2/kg bw/day) and the EE2 changes were not similar to changes when BPA treatment-related effects were observed.

Author conclusions

The authors noted that only 10 of 530 measurements of leukocyte cell populations in the thymus or spleen in BPA-treated rats were statistically different from vehicle controls. Although statistically significant effects were reported, the authors concluded that effects were not dose-dependent, generally moderate in magnitude, and transient with no persistent trend over a 1-year period (applies to spleen when multiple age groups were tested; thymus was only examined on PND 21). Authors also concluded that there were no significant changes in immune cell composition of the spleen or thymus with chronic exposure to BPA in rats.

Table 8. Summary of Statistically Significant Immune Endpoints in Li et al. (2018b)

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]					
				Vehicle	2.5	25	250	2,500	25,000
CD, 90 d	**Leukocyte Measures (Splenic)**	**% classic dendritic cells** mean ± SE, [n]	M	2.6 ± 0.2 [10]	3.8 ± 0.68* [9]	3.0 ± 0.19 [8]	2.7 ± 0.18 [10]	2.2 ± 0.32 [7]	2.8 ± 0.22 [10]
CD, 6 mo	**Leukocyte Measures (Splenic)**	**% B cells** mean ± SE, [n]	F	27.6 ± 2.47 [10]	26.6 ± 1.88 [10]	19.4 ± 0.75* [7]	24.7 ± 1.46 [9]	24.1 ± 1.75 [10]	25.7 ± 2.76 [9]
		% mature classic dendritic cells mean ± SE, [n]	M	1.7 ± 0.66 [6]	0.76 ± 0.16** [9]	1.2 ± 0.21 [8]	1.2 ± 0.24 [6]	0.79 ± 0.19** [8]	1.0 ± 0.23 [10]
	Cellularity	**Spleen cellularity** mean ± SE, (10^6 cells/mg) [n]	F	0.78 ± 0.056 [10]	0.73 ± 0.051 [10]	0.97 ± 0.063* [9]	0.76 ± 0.056 [9]	0.80 ± 0.049 [10]	0.72 ± 0.030 [9]
CD, 1 yr	**Leukocyte Measures (Splenic)**	**% antigen presenting cells** mean ± SE, [n]	M	3.4 ± 1.34 [5]	1.6 ± 0.20* [5]	1.5 ± 0.20* [3]	2.2 ± 0.40 [2]	2.7 ± 0.23 [7]	2.9 ± 0.32 [7]
		% CD8+ T cells mean ± SE, [n]	M	14.1 ± 1.08 [5]	14.6 ± 0.93 [5]	12.3 ± 0.44 [3]	14.9 ± 2.0 [2]	16.2 ± 0.83 [7]	18.5 ± 1.05* [7]
		% macrophages mean ± SE, [n]	M	16.2 ± 2.07 [5]	15.1 ± 2.39 [5]	13.0 ± 2.46 [3]	10.1 ± 0.60 [2]	10.8 ± 1.0* [7]	13.3 ± 0.80 [7]
		% NKT cells mean ± SE, [n]	M	13.1 ± 2.47 [5]	16.4 ± 0.73 [5]	12.8 ± 0.44 [3]	17.0 ± 1.55 [2]	15.6 ± 0.79 [7]	19.1 ± 0.56* [7]

CD = continuous dose; d = day; mo = month; yr = year; bw = body weight.

[1]Statistical analyses by ANOVA and Dunnett's posttest. Shading indicates statistical significance (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).

Investigational Study: Li et al. (2018a)

Methods

Animals taken from the continuous-dose treatment arm were euthanized on PND 90, 6 months, or 1 year of age. Spleens were collected in medium on ice and shipped overnight from the NCTR facility to Michigan State University for processing the following day. Splenocytes were isolated, washed, and cultured in microtiter plates in a series of assays to measure the following: (1) lymphoproliferative responses to lipopolysaccharide (LPS), pokeweed mitogen (PWM), or anti-CD3/CD28; (2) intracellular and secreted antibodies (IgM); and (3) cell activation for natural killer (NK) cells, T cells, and myeloid cells.

Findings

The authors reported several statistically significant changes in splenocytes from BPA-exposed rats from one sex, age, or BPA dose group including intracellular IgM levels, NK cell activation, and myeloid cell activation (Table 9). They also identified two effects that were reported in multiple dose groups or ages—T cell activation and the lymphoproliferative response to LPS or PWM.

BPA treatment increased T cell activation in males at 6 months (2.5, 2,500, and 25,000 µg BPA/kg bw/day dose groups) (as determined by % of $CD25^+$ T cells and/or expression level of CD25) and in the high-dose group (25,000 µg BPA/kg bw/day) at 1 year (as determined by expression level of CD25).

The authors reported alterations in the proliferative response to LPS and PWM in several BPA treatment groups and at different ages for both males and females (Table 9). There is considerable heterogeneity in the effect of BPA treatment on the proliferative response between the sexes or age groups at time of assessment although the direction of the effect (either increased and decreased proliferation) was consistent within groups by age or sex. BPA treatment was consistently associated with increased proliferation in response to both LPS (2.5, 2,500, and 25,000 µg BPA/kg bw/day dose groups) and PWM (2.5, 25, 2,500, and 25,000 µg BPA/kg bw/day dose groups) in 1-year-old males.

Ethinyl estradiol

There were some changes in the proliferative response to LPS, PWM, or anti-CD3/CD28 in male and female rats in the EE2 treatment groups. There is considerable heterogeneity in the effect of EE2 treatment on proliferation between sexes, age groups, and doses. The proliferative responses to LPS and PWM were increased in 1-year-old male rats in the EE2 treatment groups. EE2 treatment increased T cell activation in 6-month-old male rats in both dose groups (0.05 and 0.5 µg EE2/kg bw/day) (as determined by % of $CD25^+$ T cells) and in the high-dose group (0.5 µg EE2/kg bw/day) males at 1 year (as determined by expression level of CD25).

Author conclusions

The authors identified 35 of 630 measurements of splenocyte cell proliferation, activation, or IgM secretion in BPA-treated rats that were statistically different from vehicle controls. They reported that BPA treatment was associated with increased T cell activation in 6-month-old males and increased cell proliferation in 1-year-old males. However, the authors noted that neither effect was dose-dependent and neither showed a persistent trend over a 1-year period.

Furthermore, observed effects were mostly sporadic and generally moderate in magnitude. Given these findings, the authors concluded that BPA-mediated changes observed are unlikely to compromise immune competence in adult rats.

Table 9. Summary of Statistically Significant Immune Endpoints in Li et al. (2018a)

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]					
				Vehicle	2.5	25	250	2,500	25,000
CD, 90 d	Immune Function	**Activation (% MCHII+) CD11b/c cells (LPS+48 hr)** mean ± SE, [n]	F	16.4 ± 3.6 [7]	23.9 ± 4.6 [8]	17.1 ± 2.7 [8]	33.5 ± 6.4* [10]	24.7 ± 6.8 [6]	33.0 ± 6.8* [7]
		IgM Response [% Intracellular IgM^{high} (LPS activation)] mean ± SE, [n]	F	5.3 ± 1.6 [7]	7.9 ± 1.9 [8]	7.7 ± 1.2 [8]	6.5 ± 1.4 [10]	13 ± 2.9* [6]	8.8 ± 2.4 [7]
		IgM Response [% Intracellular IgM^{high} (LPS activation)] mean ± SE, [n]	M	14.6 ± 3.4 [10]	5.1 ± 1.4* [8]	12.1 ± 2.2 [8]	11.6 ± 3.9 [10]	11.2 ± 2.7 [7]	14.8 ± 3.5 [10]
		IgM Response [% Intracellular IgM^{high} (PWM activation)] mean ± SE, [n]	F	11.5 ± 1.2 [6]	15.8 ± 4.1 [4]	14.8 ± 1.1 [6]	8.8 ± 0.99 [4]	12.1 ± 0 [1]	20.5 ± 8.8** [2]
		Proliferation (count) (PWM activation) mean ± SE, [n]	F	235,511 ± 30,708 [7]	206,320 ± 21,110 [8]	217,882 ± 19,457 [8]	211,057 ± 18,563 [10]	237,113 ± 22,982 [6]	171,373 ± 20,479* [7]
CD, 6 mo	Immune Function	**Activation (%CD25+) T cells (anti-CD3/28)** mean ± SE, [n]	M	71.8 ± 3.9 [8]	75.0 ± 2.3* [9]	78.8 ± 1.4 [9]	70.8 ± 2.4 [10]	81.1 ± 1.1** [8]	78.3 ± 3.2* [10]
		Activation (%CD80+) NK cells (LPS+24 hr) mean ± SE, [n]	F	38.9 ± 3.2 [10]	30.7 ± 2.4 [10]	29.8 ± 2.7 [9]	37.4 ± 3.6 [9]	33.3 ± 3.8 [10]	28.9 ± 2.1* [9]
		Activation (%CD80+) NK cells (LPS+24 hr) mean ± SE, [n]	M	24.9 ± 3.3 [8]	39.7 ± 5.1* [9]	27.3 ± 3.3 [9]	30.1 ± 5.8 [10]	34.5 ± 4.3 [8]	32.4 ± 2.5 [10]
		Activation (%CD86+) CD172a+cells (LPS+48 hr) mean ± SE, [n]	M	69.9 ± 4.3 [6]	60.7 ± 4.3 [9]	70.9 ± 3.8 [8]	68.0 ± 6.8 [6]	51.9 ± 5.8* [8]	56.5 ± 6.0 [10]
		Activation (%CD86+) NK cells (LPS+24 hr) mean ± SE, [n]	M	49.7 ± 4.5 [8]	59.7 ± 2.2 [9]	54.6 ± 3.7 [9]	45.6 ± 3.2 [10]	61.1 ± 5.0 [8]	61.7 ± 4.1* [10]
		Activation (% MCHII+) CD172a+cells (LPS+48 hr) mean ± SE, [n]	M	51.3 ± 3.5 [6]	43.5 ± 4.4 [9]	53.2 ± 3.8 [8]	49.5 ± 8.1 [6]	31.6 ± 5.1* [8]	38.4 ± 5.9 [10]
		Activation (count) (MFI CD25) (anti-CD3/28) mean ± SE, [n]	F	24,452 ± 3,324 [10]	34,686 ± 3,280* [10]	20,011 ± 4,002 [9]	17,833 ± 2,923 [9]	25,348 ± 3,509 [10]	29,546 ± 3,776 [9]

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]					
				Vehicle	2.5	25	250	2,500	25,000
		Activation (count) (MFI CD25) (anti-CD3/28) mean ± SE, [n]	M	19,651 ± 1,889 [8]	20,084 ± 2,230 [9]	23,941 ± 1,365 [9]	19,378 ± 1,051 [10]	27,883 ± 3,105** [8]	25,619 ± 2,497* [10]
		Proliferation (count) (PWM activation) mean ± SE, [n]	F	176,360 ± 7,204 [2–10]	208,669 ± 12,140 [10]	218,964 ± 14,186** [9]	194,774 ± 11,219 [9]	234,991 ± 10,779*** [10]	208,732 ± 16,129 [9]
		Proliferation (count) (PWM activation) mean ± SE, [n]	M	366,699 ± 38,287 [6]	265,227 ± 38,051* [9]	366,658 ± 26,869 [9]	277,134 ± 24,911* [10]	242,289 ± 30,099** [8]	283,653 ± 34,363 [10]
CD, 1 yr	**Immune Function**	**Activation (% CD86+) NK cells (LPS+24 hr)** mean ± SE, [n]	M	52.0 ± 6.2 [5]	63.2 ± 5.6 [5]	39.7 ± 12.8 [3]	65.9 ± 0.50 [2]	65.6 ± 2.1 [7]	70.6 ± 3.2* [7]
		Activation (% CD86+) NK cells (LPS+48 hr) mean ± SE, [n]	M	57.2 ± 3.7 [5]	73.6 ± 3.1 [5]	78.3 ± 6.4* [3]	73.1 ± 4.0 [2]	65.2 ± 2.8 [7]	67.2 ± 2.6 [7]
		Activation (count) (MFI CD25) (anti-CD3/28) mean ± SE, [n]	F	23,800 ± 2,165 [9]	24,332 ± 3,551 [8]	17,473 ± 2,230* [8]	18,815 ± 2,142 [9]	19,242 ± 950 [8]	22,289 ± 2,101 [10]
		Activation (count) (MFI CD25) (anti-CD3/28) mean ± SE, [n]	M	16,041 ± 3,089 [5]	19,237 ± 1,143 [5]	19,153 ± 2,633 [3]	18,486 ± 3,472 [2]	18,333 ± 2,978 [7]	24,577 ± 1,673** [6]
		Proliferation (count) (anti-CD3/28) mean ± SE, [n]	F	322,217 ± 50,939 [9]	178,494 ± 39,037* [8]	243,819 ± 47,778 [8]	249,749 ± 44,163 [9]	279,333 ± 36,551 [10]	240,113 ± 51,302 [10]
		Proliferation (count) (LPS activation) mean ± SE, [n]	F	32,766 ± 8,128 [9]	49,277 ± 13,813 [8]	45,378 ± 17,788 [8]	45,825 ± 15,841 [9]	72,968 ± 19,588* [10]	40,226 ± 9,611 [10]
		Proliferation (count) (LPS activation) mean ± SE, [n]	M	22,488 ± 3,240 [5]	46,140 ± 7,771* [5]	44,139 ± 7,290 [3]	44,907 ± 15,230 [2]	43,123 ± 5,575* [7]	47,611 ± 6,043** [7]
		Proliferation (count) (PWM activation) mean ± SE, [n]	M	137,485 ± 26,631 [5]	224,294 ± 20,932** [5]	212,043 ± 5,265* [3]	177,920 ± 18,468 [2]	208,587 ± 16,672** [7]	215,175 ± 14,887** [7]

CD = continuous dose; d = day; mo = month; yr = year; bw = body weight.

[1]Statistical analyses by ANOVA and Dunnett's posttest. Shading indicates statistical significance (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$; ***, statistically significant at $p \leq 0.001$).

Chapter 4. Mammary Gland

Findings of the CLARITY-BPA core study (NTP 2018) and the investigational study from the laboratory of Ana Soto (Montévil et al. 2020) with respect to the potential effects of BPA on the mammary gland are summarized below. All individual animal data for these studies are available online at https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA . The study details, including endpoints measured and the timing of assessment, are detailed in Table 10. The statistically significant findings on the mammary gland from the core study are included in Table 11.

Table 10. Study Details and Summary of Measured Mammary Gland Endpoints

Endpoint Category	Specific Measure	Ana Soto[1] (Unpublished)	Montévil et al. (2020)[1]			NTP (2018)[2]	
		Age at Assessment					
		PND 21	PND 21	PND 90	6 mo	1 yr	2 yr
Gene Expression[3]	Mammary gland genomic DNA methylome and RNA transcriptome analysis	●	●	– –	– –	– –	– –
Morphology[4]	Mammary gland developmental scoring and histoarchitectural quantitative features	–	●	● □	● □	– –	– –
Pathology[5]	Mammary gland nonneoplastic and neoplastic lesions	–	●	● □	● □	● □	● □

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.
yr. = year; mo. = month; PND = postnatal day; bw = body weight.
[1]Female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 6 months and examined for effects.
Males were not included in the Ana Soto (unpublished) or Montévil et al. 2020 studies.
[2]Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr. or 2 yr. and examined for effects.
[3]Ana Soto (unpublished) endpoints: mammary gland (epithelium and periductal stroma) sequencing analyses of RNA transcriptomes (by RNA-seq) on PND 21; data are available at: https://doi.org/10.22427/NTP-DATA-018-00014-0001-000-5.
[4]Montévil et al. 2020 endpoints: mammary gland developmental quantitative and semiquantitative assessments on PND 21 (based on number of terminal end buds, degree of ductal branching and/or ductal budding, number of primary ducts growing from nipple, degree of lobule formation, and lateral and longitudinal growth of the gland); measurements were made using both old and new quantitative and established semiquantitative techniques. The new quantitative methods are 91 distinct measurements. Histoarchitectural features at PND 90 and 6 months were quantified (i.e., number of leading edge/internal terminal ends and incidences of lateral branching, lateral budding, alveolar budding, and lobuloalveolar development); measurements were made using nonautomatic quantitative methods.
[5]NTP 2018 endpoints: comprehensive assessment of neoplastic and nonneoplastic lesions including alveolus dilatation, atypical foci, ductal dilatation, lobular hyperplasia, adenomas, adenocarcinomas, adeno-squamous carcinomas, and fibroadenomas.
Montévil et al. 2020 endpoints: lobular or ductular alveolar dilations, lobular hyperplasia, periductular fibrosis or ductal epithelial necrosis with lymphocytic infiltration, adenomas, ductal carcinoma in situ, adenocarcinomas, and fibroadenomas.

Study Summaries

Core Study: NTP (2018)

Methods

Gross observations and histopathology of mammary glands of female and male rats were evaluated in the core study at interim sacrifice (age 1 year) and terminal sacrifice (age 2 years) in the continuous- and stop-dose arms. Mammary gland weights were not collected. Histological examination evaluated the incidence of neoplastic lesions (e.g., adenomas, adenocarcinomas, adeno-squamous carcinomas, and fibroadenomas) and nonneoplastic lesions (e.g., atypical focus, ductal dilation, lobular hyperplasia, and alveolus dilation).

Findings

At 2 years in stop-dose females, the incidence of adenocarcinoma and the combined incidence of adenoma or adenocarcinoma were higher in the 2.5 µg BPA/kg bw/day continuous-dose group compared to controls (Poly-3) (Table 11). Fibroadenomas are commonly seen in females of this rat strain at 1 and 2 years of age, and they occurred with a decreasing trend in BPA-exposed groups (Poly-3). Although neoplastic lesions were reported in the continuous-dose arm, they were not different in incidence across dose groups in female or male rats at either 1 or 2 years.

In continuous-dose females, the incidence of atypical foci was higher in the 2.5 µg BPA/kg bw/day group compared to controls at 1 and 2 years (RTE). The incidence of duct dilatation was higher in the 25 µg BPA/kg bw/day group than in controls at 1 year (RTE), but was lower at 2 years (RTE, JT/SW). At 2 years, the incidence and severity of lobular hyperplasia was lower in continuous-dose females by trend test (Poly-3) and pairwise comparisons at 25 (Poly-3 and RTE) and 2,500 µg BPA/kg bw/day (RTE). In continuous-dose males, the single significant observation was a higher incidence of alveolus dilatation in the 2.5 µg BPA/kg bw/day exposure group at 2 years (Poly-3 and RTE).

In the stop-dose BPA females, the incidence of duct dilation at 1 year was lower by trend analysis (CAFE, JT/SW, and RTE) as well as by pairwise comparison at 250 (RTE) and 25,000 µg BPA/kg bw/day (JT/SW and RTE). Similarly, at 2 years, the incidence of duct dilatation was lower than in controls at all BPA doses (Poly-3 [2.5 and 2,500 µg BPA/kg bw/day only], JT/SW, and RTE [2.5–2,500 µg BPA/kg bw/day only]). Also, the incidence of lobular hyperplasia in stop-dose BPA females was lower at 1 year by trend analysis (Poly-3) and by pairwise comparison in the 25 and 2,500 µg BPA/kg bw/day dose groups (RTE). At 2 years, alveolus dilatation was lower in females treated with 2,500 µg BPA/kg bw/day (RTE). In stop-dose males, the incidence of alveolus dilatation was lower at all BPA doses at 2 years by trend analysis (JT/SW and RTE) and by pairwise comparison (JT/SW, RTE; Poly-3 at 25 and 2,500 µg BPA/kg bw/day only).

Ethinyl estradiol

In continuous-dose females, a negative trend for the incidence of fibroadenomas was reported in EE2-dosed females at 1 year (CAFE). At 2 years, the incidence of adenocarcinoma was higher in EE2-exposed females by trend analysis and pairwise comparison of 0.5 µg EE2/kg bw/day compared to controls (Poly-3). Regarding nonneoplastic lesions in continuous-dose females at 1 year, higher incidences of duct dilatation and lobular hyperplasia were observed in EE2-

exposed females by trend analysis and pairwise comparison of 0.5 μg EE2/kg bw/day compared to controls (CAFE, JT/SW, and RTE). At 2 years, higher incidences of duct dilatation and alveolus dilatation were observed in EE2-exposed females by trend analysis and pairwise comparison of 0.5 μg EE2/kg bw/day versus controls (Poly-3, JT/SW, and RTE). In addition, a higher incidence of galactocele was observed in continuous-dose females at 1 and 2 years by trend analyses (CAFE and Poly-3, respectively). In continuous-dose males, the only significant result was a lower incidence of duct dilatation at 2 years observed by trend analysis and by pairwise comparison of 0.5 μg EE2/kg bw/day versus controls (Poly-3, JT/SW, and RTE).

Author conclusions

Although the incidence of mammary adenocarcinoma, or the combined incidence of adenomas and adenocarcinomas, in the stop-dose females exposed to 2.5 μg BPA/kg bw/day was increased as compared to vehicle controls, the authors concluded that "it was unlikely plausible that these were BPA treatment-related lesions since the elevation in lesions occurred only in the low-dose treatment group and only in the stop-dose arm of the study." Further, the authors stated that they only had "limited historical control data for this strain of rat" and that "it was difficult to envision a mechanism where stop-dose animals would develop mammary adenocarcinoma while continuous-dose animals would not" (NTP 2018).

Investigational Study: Montévil et al. (2020)

Methods

Mammary glands were collected from continuous- and stop-dose female rats at PND 21, PND 90, and 6 months (n = 8–10 per treatment group per time point). One female per litter was necropsied per treatment group per time point. At PND 90, all animals were predicted to be in estrus at time of sacrifice as determined by vaginal smears taken the day before necropsy and postmortem pathology examination. Right and left fourth inguinal mammary glands were removed and weighed individually. The excised right fourth inguinal mammary glands were whole mounted to a charged slide and fixed in 70% ethanol, while the contralateral left glands were placed in a histological cassette and fixed in 70% ethanol for histopathology. Tissues were shipped to the Soto laboratory for processing and evaluation. Whole mounts were stained with carmine and processed for three-dimensional (3D) autofluorescence imaging, and contralateral glands were paraffin embedded and processed for histoarchitectural features and histopathology. Histopathology was assessed by a board-certified pathologist. In addition to the chronic study (CLARITY-BPA) mammary gland tissues, this study evaluated mammary gland tissues collected from female rats at PND 21 and PND 90 in a subchronic study of BPA described in Delclos et al. (2014); the subchronic study was conducted prior to the CLARITY-BPA study and utilized the same rat strain, continuous dosing study design, and a similar dose range of BPA and positive control EE2 exposure groups as the CLARITY-BPA study animals.

The mammary gland samples were examined using three morphometric scoring techniques, two of which are established methods: semiquantitative mammary gland scoring and nonautomatic quantitative morphometric analysis (Davis and Fenton 2013) and a new method of automatic quantitative morphometric analysis. Slides from whole mounts of treated and control animals were independently evaluated by two trained investigators who scored them in a blinded manner. Briefly, for the semi-automatic quantitative method, whole mounts of PND 21 females were viewed under a stereo microscope and given a morphological developmental score from 1 to 7

based on (a) the number of terminal end buds relative to the number of duct ends, (b) the degree of ductal branching and/or ductal budding, (c) the number of primary ducts growing from the point of attachment, (d) the degree of lobule formation, and (e) the lateral and longitudinal growth of the gland (extension). For the standard nonautomatic quantitative assessment, images of mammary glands of PND 90 and 6-month females were captured on a stereo microscope and visually scored for countable morphological parameters, including number of leading edge/internal terminal ends and incidences of lateral branching, lateral budding, alveolar budding, and lobuloalveolar development. Additionally, epithelial density was quantitatively determined by a new automatic morphometric analysis. For the new automatic morphometric analysis, mammary gland samples from PND 21 females were imaged with a Zeiss confocal microscope and examined using a customized automatic method described in detail in Montévil et al. (2020). This new method used optical sections to construct a 3D image of the carmine autofluorescence staining visualized by a confocal microscope. The separate images were computationally stitched together, and the morphological features were measured using ImageJ, an open source, JAVA-based software program used to measure multidimensional images. Ninety-one structural endpoints as well as body weight, mammary gland weight, and manual assessment of the number of terminal endbuds were measured for each animal. Because of the large number of correlated endpoints for any given animal, statistical analyses included principal component analysis (PCA) and permutation tests.

For the genomic studies, female mammary tissues were collected at PND 21 (n = 4 animals per group, 1 animal per litter) for RNA transcriptome endpoints. In brief, right and left 4th and 5th inguinal mammary glands were collected under RNase-free conditions, flash frozen, and shipped overnight to the Soto laboratory. The periductal stroma (i.e., the outer approximately 100-µm boundary of the epithelium) and epithelial ducts were collected separately by laser capture microdissection. Samples were analyzed by RNA-seq.

Findings

The results from individual endpoint evaluations of CLARITY-BPA mammary gland tissues described in this section did not reach statistical significance and therefore are not included in the summary table of statistically significant findings. A narrative summary of the findings is presented below followed by the results of an integrated analysis of 94 measurements of mammary gland characteristics.

The semiquantitative analysis of the mammary gland tissues from the chronic and subchronic studies revealed no significant differences between control and BPA dose groups at PND 21 for body weight, excised mammary gland weight, and the semiquantitative score of mammary gland development. However, at PND 90, accelerated mammary gland development was observed in the 2.5 µg BPA/kg bw/day dose group relative to controls in PND 90 female rats in estrus from the subchronic study of BPA. In addition, there was a decrease in the mammary gland developmental score between the 25 and 260 µg BPA/kg bw/day dose groups at PND 90 in the subchronic study.

Global analysis: 25–250 µg BPA/kg bw/day as a breaking point

Exploratory analyses. Using 94 measurements (91 structural features plus animal weight, mammary gland weight, and manual assessment of terminal end bud morphology), PCA analysis of PND 21 tissues from the CLARITY-BPA (chronic) study suggested that BPA effects were not

similar to EE2, and that BPA treatment was associated with different morphological changes than that of 0.5 µg EE2/kg bw/day. Regarding the BPA data, the authors stated, "Graphically, the [dose-]response seemed nonmonotonic with a breaking point between 25 and 250 BPA; 2.5BPA and 25BPA are close, whereas 250BPA is very different from 25BPA and control, and high BPA doses are roughly between 25BPA and 250BPA" (Montévil et al. 2020). Similar patterns were observed when the EE2 data were excluded from the PCA analysis.

Hypothesis formulation. The chronic study PND 21 results were used as the basis to formulate statistical hypotheses by using the permutation test. These results suggested that the hypothesis H0 (i.e., BPA exposure has no effect) should be rejected in favor of the hypothesis H1 (that 25–250 µg BPA/kg bw/day is the location of the largest change for a larger number of variables than in H0).

Confirmatory tests. The remaining four independent data sets (PND 90 continuous-dose, PND 90 stop-dose, 6 month continuous-dose, 6 month stop-dose animals) were used for a confirmatory analysis, and they revealed similar changes to those observed in the chronic study PND 21 results. Thus, the H0 could be safely rejected and the alternative H1 was adopted. The authors concluded that, "A significantly high number of variables had their largest change between 25BPA and 250BPA, and, accordingly, this interval was the locus of a modified response to BPA" (see Figure 7) (Montévil et al. 2020). Additionally, the BPA response was different from the EE2 effect for numerous features.

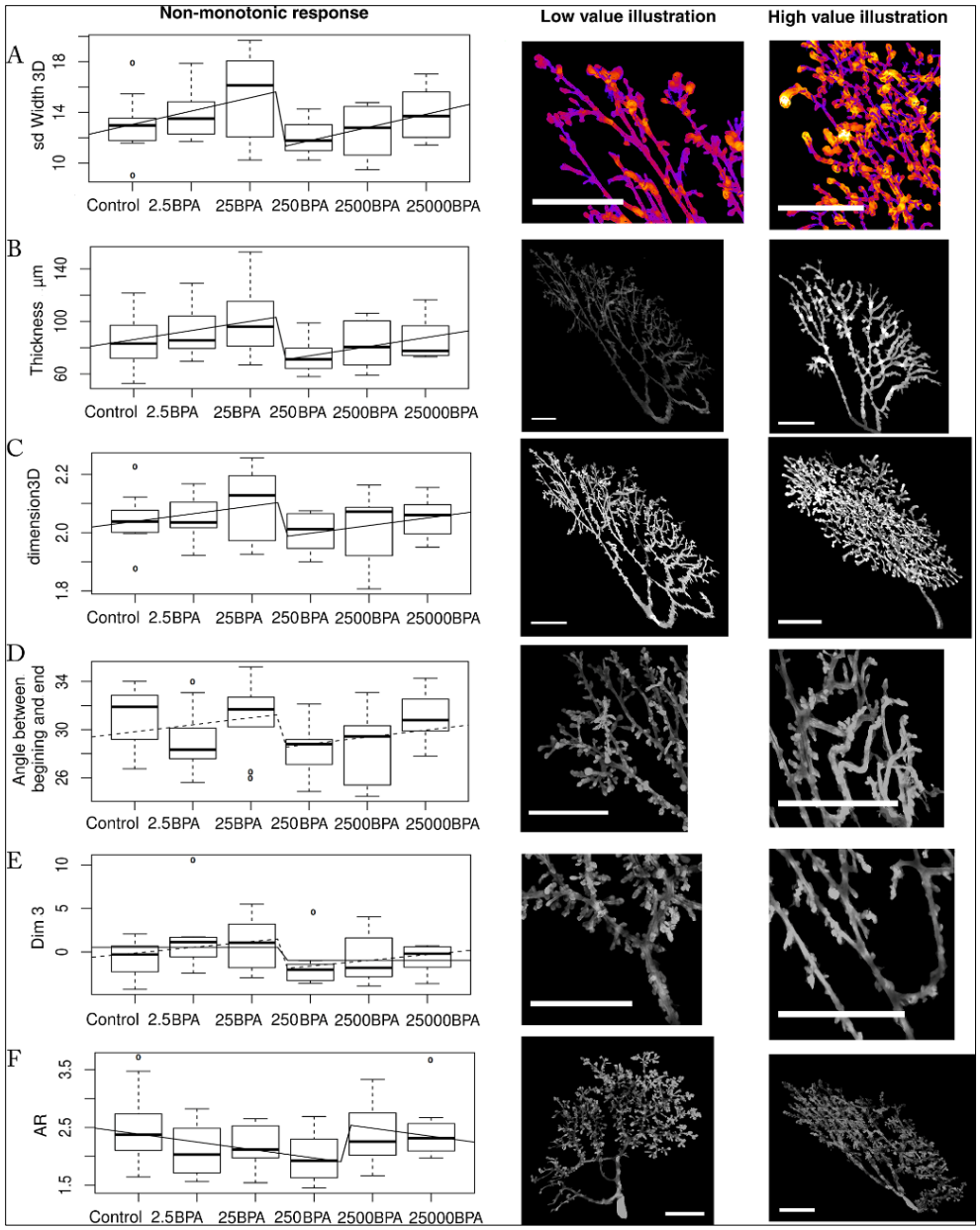


Figure 7. Nonmonotonic Responses to BPA Doses in PND 21 Female Rat Mammary Glands and Illustration of Corresponding Morphological Features

The midline represents the median, the box represents the quartiles above and below the median and the whiskers represent the two other quartiles, excluding outliers. Graphs on the left represent mean and standard deviation for each dose and the fit with the combination of a linear and step function. Left panels are representative images of a low value, right panels illustrate high values. Scale bars = 2 mm. All features but the aspect ratio (F) show a break between 25 µg/kg bw/day BPA and 250 µg/kg bw/day BPA. In (F) the break is between 250 µg/kg bw/day BPA and 2,500 µg/kg bw/day BPA. (A) Mean variation of ductal thickness: the gland on the right has many structures that have both thin and thick parts while the gland on the left has more regular structures. (B) Mean thickness of the epithelium: the brightness in the pictures is proportional to the local thickness of the points of the gland. (C) Fractal dimension in 3D. The gland on the right grows more conspicuously in the third dimension than in the left figure. (D) Angle between the beginning and the end of ducts: ducts are straighter on the left and turn more on the right. (E) Third dimension from PCA. (F) Aspect ratio (AR). A large AR leads to an elongated gland while a low AR means that the gland is round. Low doses of BPA increase the roundness of glands and high doses lead to an AR similar to control. [Reprinted with permission from Montévil et al. (2020).]

Ethinyl estradiol

At PND 21, treatment with EE2 at 0.5 µg/kg bw/day significantly advanced glandular development compared to vehicle controls. Regarding the chronic study PND 21 data set, the effects of 250 µg BPA/kg bw/day and 0.5 µg EE2/kg bw/day with respect to the vehicle control, and with respect to each other, varied depending on the specific measurement. In some instances, the responses to BPA and EE2 were similar; in others, they were different and even opposite.

The data from the PND 21 mammary gland genomic DNA methylome and RNA transcriptome analysis were not included in the paper (Montévil et al. 2020) due to sample degradation (Ana Soto, personal communication); however, the unanalyzed data from these studies are available at: https://doi.org/10.22427/NTP-DATA-018-00014-0001-000-5.

Author conclusions

The authors concluded that the mammary gland is a "sensitive target for endocrine disruption" and that "measurable effects manifest at low levels of exposure to endocrine disruption" (Montévil et al. 2020). Further, the authors concluded that BPA demonstrated nonmonotonic responses as determined by both semiquantitative and quantitative methods of mammary gland development. The authors stated that "At all time points observed, lower doses resulted in larger effects, consistent with the core study (NTP 2018), which revealed a significant increase of mammary adenocarcinoma incidence in the stop-dose animals at the lowest BPA dose tested" (Montévil et al. 2020).

The authors concluded that, using semiquantitative and quantitative methods, BPA induced effects in the mammary gland that were different from those of EE2. In addition to the occurrence of low-dose effects and nonmonotonicity, the authors stated that this study shows that nonmonotonicity can have different shapes than the inverted "U-shaped" (or quadratic) responses traditionally associated with references to nonmonotonicity. The authors commented that, although several features measured by the quantitative method are not similar to the features used for the scoring method, they reveal comparable physiological information (i.e., the fractal dimension assesses the complexity of the ductal system, and the mean variation of ductal thickness is associated with budding). Thus, they are indicators of response to hormones and endocrine disruptors.

Finally, the authors (Montévil et al. 2020) concluded, "These results show the importance of establishing and using statistical methods appropriate for nonmonotonic responses. Linear models are a powerful tool to provide evidence of a causal relationship because they quantitatively relate the changes of a putative cause with the one of the effects. Moreover, linear responses to small causes are a common mathematical property albeit not universal. Therefore, exhibiting a linear response is a powerful method to provide empirical evidence of a causal relationship in a given context. However, this method is blind to nonmonotonic responses. The latter are common in endocrinology because the putative causes are involved in multilevel, complex regulations due to the evolutionary history of hormones and their functions. In this context, a more appropriate way to show the presence of causation is to show the prevalence of a specific nonmonotonic pattern, here a breaking point between 25BPA and 250BPA."

Table 11. Summary of Statistically Significant Mammary Gland Endpoints

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]						Reference
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
CD, 1 yr	**Pathology[4]**	**Atypical focus** incidence[5]/n, % incidence (severity profile)	F	NS	0/23, 0% (-)	3/22, 14%^ (1|2|0|0)	2/22, 9% (0|2|0|0)	2/24, 8% (2|0|0|0)	0/20, 0% (-)	0/24, 0% (-)	NTP, 2018
		Duct dilatation incidence/n, % incidence (severity profile)	F	NS	2/23, 9% (1|1|0|0)	2/22, 9% (0|0|0|2)	7/22, 32%^^ (2|4|1|0)	1/24, 4% (0|0|1|0)	2/20, 10% (1|0|1|0)	2/24, 8% (1|0|1|0)	NTP, 2018
SD, 1 yr		**Duct dilatation** incidence/n, % incidence (severity profile)	F	↓*,#,^	4/20, 20% (2|1|1|0)	2/22, 9% (1|1|0|0)	1/20, 5% (0|1|0|0)	1/22, 4%N^ (1|0|0|0)	1/20, 5% (0|1|0|0)	1/22, 4%N#,^ (1|0|0|0)	NTP, 2018
		Lobular hyperplasia incidence/n, % incidence (severity profile)	F	NS	15/20, 75% (10|4|1|0)	12/22, 54% (8|3|1|0)	8/20, 40%N*,#6^ (5|1|2|0)	12/22, 54% (8|3|1|0)	7/20, 35%N*,#6,^ (4|3|0|0)	12/22, 54% (6|4|2|0)	NTP, 2018
CD, 2 yr		**Atypical focus** incidence/n, % incidence (severity profile)	F	NS	2/50, 4% (0|2|0|0)	7/48, 15%^ (3|2|2|0)	1/46, 2% (0|1|0|0)	5/49, 10% (0|5|0|0)	3/50, 6% (1|2|0|0)	3/46, 6% (1|2|0|0)	NTP, 2018
		Alveolus dilatation incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	M	NS	8/50, 16% 8/35.0, 23% (0|5|3|0)	17/48, 35%^ 17/36.8, 46%* (0|10|6|1)	11/48, 23% 11/34.7, 32% (0|4|6|1)	10/50, 20% 10/34.6, 29% (0|8|2|0)	11/50, 22% 11/36.1, 30% (0|7|4|0)	9/45, 20% 9/29.5, 31% (0|8|0|1)	NTP, 2018
		Duct dilatation incidence/n, % incidence (severity profile)	F	NS	15/50, 30% (0|10|5|0)	16/48, 33% (0|12|2|2)	7/46, 15%N#5,^ (0|6|1|0)	9/49, 18% (0|6|3|0)	9/50, 18% (0|4|5|0)	14/46, 30% (2|9|3|0)	NTP, 2018
		Lobular hyperplasia incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	F	↓*	43/50, 86% 43/45.4, 95% (1|8|13|21)	41/48, 85% 41/42.5, 96% (1|7|11|22)	30/46, 65%N#5,^^ 30/36.7, 82%N* (1|7|10|12)	38/49, 78%N#5 38/43.7, 87% (0|7|17|14)	40/50, 80%N^ 40/43.2, 93% (1|14|11|14)	37/46, 80% 37/43.4, 85% (4|9|5|19)	NTP, 2018
SD, 2 yr		**Adenocarcinoma** Poly-3 incidence/ adjusted n, % incidence	F	NS	3/32.3, 9%	11/33.3, 33%*	5/32.1, 16%	7/35.4, 20%	9/36.6, 25%	5/32, 16%	NTP, 2018
		Adenoma or adenocarcinoma Poly-3 incidence/ adjusted n, % incidence	F	NS	4/33, 12%	12/33.3, 36%*	5/32.1, 16%	9/35.9, 25%	9/36.6, 25%	6/32.8, 18%	NTP, 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
		Alveolus dilatation incidence/n, % incidence (severity profile)	F	NS	8/50, 16% (0|4|4|0)	4/50, 8% (0|4|0|0)	4/48, 8% (0|2|2|0)	8/49, 16% (0|6|2|0)	3/50, 6%$^{N\wedge}$ (1|2|0|0)	7/46, 15% (1|6|0|0)	NTP, 2018
		Alveolus dilatation incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	M	↓$^{N\#,\wedge}$	15/49, 31% 15/38.9, 39% (0|11|4|0)	7/48, 15%$^{N\#,\wedge}$ 7/36.2, 19% (0|4|1|2)	6/47, 13%$^{N\#,\wedge}$ 6/35.0, 17%^{N*} (0|3|3|0)	7/50, 14%$^{N\#,\wedge}$ 7/31.7, 22% (0|4|3|0)	6/49, 12%$^{N\#\#,\wedge\wedge}$ 6/37.2, 16%^{N*} (0|5|1|0)	7/45, 16%$^{N\#,\wedge}$ 7/29.1, 24% (0|5|2|0)	NTP, 2018
		Duct dilatation incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	F	NS	16/50, 32% 16/37.0, 43% (0|9|4|3)	5/50, 10%$^{N\#\#,\wedge\wedge}$ 5/34.5, 14%^{N**} (0|5|0|0)	9/48, 19%$^{N\#,\wedge}$ 9/33.7, 27% (2|4|2|1)	9/49, 18%$^{N\#,\wedge}$ 9/35.3, 25% (0|6|3|0)	7/50, 14%$^{N\#\#,\wedge\wedge}$ 7/36.4, 19%^{N*} (0|6|1|0)	11/46, 24%$^{N\#}$ 11/36.2, 30% (0|7|4|0)	NTP, 2018
		Fibroadenoma or adenocarcinoma Poly-3 incidence/ adjusted n, % incidence	F	↓*	45/47.8, 94.2%	46/47.7, 96.5%	38/42.8, 88.9%	43/47, 91.5%	38/46.3, 82.1%	38/41.5, 91.6%	NTP, 2018
		Fibroadenoma Poly-3 incidence/ adjusted n, % incidence	F	↓*	43/47.7, 90%	45/47.5, 95%	37/42.2, 88%	42/46.2, 91%	36/45.5, 79%	34/40.6, 84%	NTP, 2018

CD = continuous dose; SD = stop dose; bw = body weight.

[1]Shading indicates statistical significance. Statistical tests varied by reference (see subsequent footnotes).

[2]↑ = positive trend, ↓ = negative trend, NS = no significant trend.

[3]The results of the 90-day (pilot) study, which evaluated four doses of BPA at 2.5, 25, 260, and 2,700 µg/kg bw/day, are presented here. Data were analyzed by Kruskal-Wallis nonparametric tests and a Dunn's post hoc comparison of vehicle control versus treated glands.

[4]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected. Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.

Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.

Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests. All pairwise tests were one-sided and not corrected for multiple comparisons.

Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs.

Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; N superscript, negative trend, or negative relative to control.

[5]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.

[6]SW test was nonsignificant due to the assumption of monotonicity; thus, this JT/SW data analysis result was not summarized in the CLARITY-BPA integrated report.'

Chapter 5. Ovary

Summarized below are findings of the CLARITY-BPA core study (NTP 2018) and the investigational study by the laboratory of Jodi Flaws (Patel et al. 2017) with respect to the potential effects of BPA on the ovary. All individual animal data for these studies are available online at https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA . The study details, including endpoints measured and timing of assessment, are detailed in Table 12. The statistically significant findings from the investigational study and the statistically relevant findings from the core study on related endpoints are included in Table 13.

Table 12. Study Details and Summary of Measured Endpoints for Ovary

Endpoint Category	Specific Measure	Patel et al. (2017)[1]			NTP (2018)[2]		Patel et al. (2017)[1]		NTP (2018)[2]	
		Age at Assessment								
		PND 1	PND 21	90 d	16 ± 2 wk	20–22 wk	6 mo	1 yr	1 yr	2 yr
Biochemical[3]	Hormone	–	●	● □	– –	– –	● □	● □	– –	– –
Female Reproductive Function[4]	Estrous cyclicity	–	–	– –	● □	●[5] □[5]	– –	– –	– –	– –
Organ Weight[6]	Ovary weight	–	–	– –	– –	– –	– –	– –	● □	– –
Pathology[7]	Ovary, folliculogenesis	●	●	● □	– –	– –	● □	● □	– –	– –
	Ovary, nonneoplastic lesions	–	–	– –	– –	– –	– –	– –	● □	● □

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.
yr = year; d = day; mo = month; wk = week; bw = body weight; PND = postnatal day.
[1]Female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at PND 1, PND 21, 90 d, 6 mo, or 1 year and examined for effects.
[2]Female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr or 2 yr and examined for effects. Estrous cycle analysis was conducted at 16 ± 2 wk of age. Time of onset of aberrant estrous cycles in aging females was monitored monthly, starting one month after the collection of the 14 consecutive vaginal smears to evaluate the estrous cycle (approximately 20–22 wk of age) and up to approximately 1 yr.
[3]Patel et al. 2017 endpoint list: estradiol levels; progesterone levels.
[4]NTP 2018 endpoint list: estrous cyclicity: abnormal (extended) diestrus, abnormal (extended) estrus, abnormal (extended) proestrus, abnormal cycling combined [16 ± 2 wk].
[5]Aberrant estrous cycles: time of onset [starting 20–22 wk and examined monthly through approximately 1 yr].
[6]NTP 2018 endpoint list: ovary weight: absolute, relative to body weight, relative to brain weight.
[7]NTP 2018 endpoint list: comprehensive assessment of neoplastic and nonneoplastic lesions, including: ovary: corpora lutea depletion, corpora lutea cysts, interstitial cell hypertrophy, periovarian tissue cysts [1 yr]; atrophy, cystic bursa, follicular cysts, Sertoliform hyperplasia [1 and 2 yr].
Patel et al. 2017 endpoint list: number of primordial follicles [all time points]; number of follicles in apoptotic germ cells and healthy germ cells [PND 1]; number of follicle types (antral follicles, preantral follicles, primary follicles); number of total healthy follicles [all time points except PND 1].

Study Summaries

Core Study: NTP (2018)

Methods

The endpoints evaluated in the core study related to ovary were ovary weights evaluated at 1 year and histopathology evaluated at 1 and 2 years. Analyses of estrous cyclicity and onset of aberrant estrous cycles were evaluated at 16 ± 2 weeks of age and in aging females (around 1 year of age), respectively.

Findings

Ovary

In the continuous-dose arm, interstitial cell hypertrophy was greater at 1 year in the 2,500 µg BPA/kg bw/day dose group (RTE) (Table 13). In addition, corpora luteal depletion, corpora luteal cysts, and interstitial cell hypertrophy occurred with positive trends across BPA exposure groups relative to controls (CAFE) at 1 year, and the trend for interstitial cell hypertrophy was also significant (JT/SW and RTE).

In the stop-dose arm, negative trends for absolute and adjusted ovary weight occurred at 1 year, and the highest dose (25,000 µg BPA/kg bw/day) resulted in a 13% decrease in ovary weight compared to control. Ovarian follicle cysts were also greater in the 25,000 µg BPA/kg bw/day dose group compared to controls and occurred with positive trends at 1 year (CAFE).

Estrous cyclicity

Females at 16 ± 2 weeks of age underwent 14 consecutive daily vaginal smears to determine abnormalities in the estrous cycle. There were no significant differences in estrous cycle length or percentage of abnormal estrous cycles (i.e., extended periods of any cycle stage) among the BPA stop- or continuous-dose groups and the vehicle controls.

In aging females (approximately 1 year), there was a delay in the onset of aberrant cycles in the 2,500 µg BPA/kg bw/day dose group in the stop-dose arm (57 weeks versus 42 weeks in vehicle control). Although a formal statistical analysis was not performed, there were apparent differences between the vehicle controls in the continuous- and stop-dose arms, with the estimated median time of onset of aberrant cycling appearing to be markedly shorter in the stop-dose control group than in the continuous-dose control group (42 weeks versus 57 weeks).

Ethinyl estradiol

All significant pairwise comparisons of EE2 on ovary and estrous cycle endpoints occurred at the higher dose (0.5 µg/kg bw/day). Exposure to EE2 resulted in reduced absolute and adjusted ovary weights at 1 year. There were also increases in ovarian atrophy (CAFE, JT/SW, and RTE), follicular cysts (CAFE), corpora lutea depletion (CAFE), and interstitial cell hypertrophy (CAFE, JT/SW, and RTE) compared to controls, and these effects, along with bursal cysts (CAFE) also occurred with positive trends at 1 year. At 2 years, ovarian atrophy was increased by pairwise comparison and trend test (JT/SW and RTE), and ovarian cysts and bursal cysts occurred with positive survival adjusted trends (Poly-3). Abnormal estrous cyclicity (specifically extended estrus) and earlier onset of aberrant cycles were also reported in animals analyzed at 16 ± 2 weeks and approximately 1 year, respectively (CAFE).

Author conclusions

The authors concluded that, "there were trends in interim sacrifice animals for depletion of corpora lutea and interstitial cell hypertrophy in the continuous-dose arm and a trend and increase in follicular cysts at 25,000 μg BPA/kg bw/day in stop-dose BPA groups. The magnitude of the increase in follicular cysts at the high BPA dose and the trend evident in the two highest BPA groups suggests that this is a treatment-related effect, although the lack of effect on this endpoint in the continuous-dose animals cannot be readily explained. In the ovaries of interim sacrifice high-dose EE2 females, there was a 100% incidence of cystic follicles" (NTP 2018). Study authors concluded that BPA did not have adverse effects on the estrous cycle.

Investigational Study: Patel et al. (2017)

Methods

Ovaries and serum from animals at PND 1, PND 21, PND 90, 6 months, and 1 year were collected from the continuous- and stop-dose arms. One ovary from each animal was fixed in Dietrich's solution and the other was snap frozen in liquid nitrogen prior to shipment to the Flaws laboratory at the University of Illinois. The fixed ovaries were used to assess ovarian follicle numbers. Primordial, primary, preantral, and antral follicle types as well as the percentage of healthy follicles were evaluated. Frozen ovaries were kept for later study. Serum estradiol and progesterone levels were measured.

Findings

Daily exposure to 2.5 and 250 μg BPA/kg bw/day decreased the numbers of primordial, primary, preantral, and total healthy follicle numbers at PND 21 (Table 13). Exposure to 2,500 and 25,000 μg BPA/kg bw/day reduced estradiol levels at 1 year, and several BPA treatments (2.5, 25, and 250 μg/kg bw/day) caused a borderline significant reduction in estradiol levels.

Ethinyl estradiol

Exposure to EE2 at 0.5 μg/kg bw/day resulted in a decrease in preantral and antral follicles at PND 21, PND 90, and/or 6 months and an increase in primary follicles at 1 year. EE2 exposure elevated serum progesterone concentrations at PND 21 but lowered serum progesterone and estradiol concentrations at 6 months and 1 year, respectively.

Author conclusions

The authors concluded that, "…BPA exposure at some doses and time points alters follicle numbers and sex steroid levels in female rats." Furthermore, because the observed effects of BPA exposure on follicle numbers and hormone levels were different from those observed for EE2, the authors concluded that, "…it is highly suggested that BPA is acting on the rat ovaries in a manner different from EE2" (Patel et al. 2017).

Table 13. Summary of Statistically Significant Endpoints for Ovary

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
CD, PND 21	**Pathology[3]**	**Preantral follicles: follicle number** mean ± SE [n]	–	52.1 ± 6.1 [8–10]	30.2 ± 3* [8–10]	43.9 ± 4.8 [8–10]	38.1 ± 6.9 [8–10]	47.5 ± 4.9 [8–10]	45.2 ± 6.2 [8–10]	Patel et al., 2017
		Primary follicles: follicle number mean ± SE [n]	–	79.9 ± 16.4 [8–10]	29.2 ± 2.4* [8–10]	82.8 ± 13.2 [8–10]	33.8 ± 5.1* [8–10]	69 ± 19.3 [8–10]	79.1 ± 9.7 [8–10]	Patel et al., 2017
		Primordial follicles: follicle number mean ± SE [n]	–	281 ± 21.7 [8–10]	188 ± 26.5 [8–10]	302 ± 47 [8–10]	172 ± 21.8* [8–10]	277 ± 46.2 [8–10]	273 ± 17 [8–10]	Patel et al., 2017
		Total healthy follicles: follicle number mean ± SE [n]	–	513 ± 45.3 [8–10]	330 ± 35.7* [8–10]	505 ± 68.8 [8–10]	315 ± 33.7* [8–10]	476 ± 70.1 [8–10]	476 ± 29.5 [8–10]	Patel et al., 2017
SD, 20–22 wk	**Female Reproductive Function[4]**	**Aberrant estrous cycles: time of onset** median (95% lower, upper CI), (wk) [n]	NS	41.9 (41.3, 51.7) [26]	51.7 (36.9, 57.0) [26]	46.8 (41.9, 56.9) [26]	51.9 (41.9, 56.9) [26]	56.9 (51.7, 66.6)* [25]	52.1 (41.9, 61.9) [26]	NTP, 2018
CD, 1 yr	**Biochemical[3]**	**Estradiol levels** mean ± SE, (pg/mL) [n]	–	46.1 ± 1.4 [3–9]	28.1 ± 8.8 [3–9]	27 ± 2.9 [3–9]	28.8 ± 4.3 [3–9]	20.6 ± 5.3* [3–9]	23.7 ± 3.5* [3–9]	Patel et al., 2017
	Pathology[5]	**Corpora lutea depletion** incidence[6]/n, % incidence	↑*	4/23, 17%	4/22, 18%	7/22, 32%	4/24, 17%	8/20, 40%	9/24, 38%	NTP, 2018
		Cystic corpora lutea incidence/n, % incidence	↑*	0/23, 0%	0/22, 0%	0/22, 0%	0/24, 0%	2/20, 10%	1/24, 4.2%	NTP, 2018
		Interstitial cell hypertrophy incidence/n, % incidence (severity score)	↑*,#,^	4/23, 17% (2)	4/22, 18% (2.2)	6/22, 27% (2.7)	3/24, 12% (3.3)	8/20, 40%[#7^] (2.2)	9/24, 38% (2)	NTP, 2018
SD, 1 yr	**Organ Weight[8]**	**Ovary weight: absolute** mean ± SE, (mg) [n]	↓*	157 ± 6 [20]	149 ± 4 [22]	148 ± 6 [20]	147 ± 4 [22]	147 ± 6 [20]	136 ± 5* [20]	NTP, 2018
		Ovary weight: relative to body weight mean ± SE, (mg/g) [n]	↓*	0.34 ± 0.02 [20]	0.33 ± 0.01 [22]	0.35 ± 0.02 [20]	0.33 ± 0.02 [22]	0.33 ± 0.01 [20]	0.31 ± 0.01 [20]	NTP, 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
		Ovary weight: relative to brain weight mean ± SE, (mg/g) [n]	↓*	75 ± 3 [20]	71 ± 2 [22]	71 ± 3 [20]	72 ± 2 [22]	70 ± 3 [20]	66 ± 3* [20]	NTP, 2018
	Pathology[5]	**Follicular cysts** incidence/n, % incidence	↑***	5/20, 25%	6/22, 27%	4/20, 20%	7/22, 32%	11/20, 55%	18/22, 82%***	NTP, 2018

CD = continuous dose; SD = stop dose; PND = postnatal day; bw = body weight.
[1]Shading indicates statistical significance. Statistical tests varied by reference (see subsequent footnotes).
[2]↑ = positive trend; ↓ = negative trend; NS = no significant trend; hyphen (–) = not evaluated.
[3]Statistical analyses by ANOVA and Dunnett's two-sided t-test (*, statistically significant at $p \leq 0.05$).
[4]Examined in terminally sacrificed dose group (sacrificed at 2 years). One month after the collection of the 14 consecutive vaginal smears to evaluate the estrous cycle (approximated as 20–22 wk); an accelerated failure time model assuming a lognormal distribution was used for analysis, and multiple comparisons were adjusted using Holm's method for treatment comparisons to the control (*, statistically significant at $p \leq 0.05$).
[5]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected.
Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.
Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.
Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests. All pairwise tests were one-sided and not corrected for multiple comparisons.
Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs.
Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; ***, ###, ^^^, statistically significant at $p \leq 0.001$; N superscript, negative trend, or negative relative to control.
[6]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.
[7]SW test was nonsignificant due to the assumption of monotonicity; thus, this JT/SW data analysis result was not summarized in the CLARITY-BPA integrated report.
[8]Organ weights were collected at interim sacrifice (1 yr) only. ANOVA (followed by Dunnett's test) was used to determine the effect of treatment on absolute organ weight. Separate ANOCOVA (followed by Dunnett's test) was used to determine the effect of treatment on organ weight adjusted for body weight or brain weight (*, statistically significant at $p \leq 0.05$).

Chapter 6. Penile Function

Findings of the CLARITY-BPA core study and investigational study by Gonzalez-Cadavid (Unpublished) with respect to endpoints reflective of penile function are reviewed. The CLARITY-BPA core study did not include assessments with respect to the potential effects of BPA on penile function. A brief discussion of methods and a link to the raw data from the unpublished penile function study carried out in the Gonzalez-Cadavid laboratory are provided. The study details, including endpoints measured and timing of assessment, are detailed in Table 14.

Table 14. Study Details and Summary of Measured Penile Function Endpoints

Endpoint Category	Specific Measure	Gonzalez-Cadavid (Unpublished)[1]
		Age at Assessment
		6 mo
Organ Weight	Penile shaft weight, absolute	●
Pathology[2]	Penile corpora cavernosa	●
	Penile tunica albuginea	●
Male Reproductive Function[3]	Erectile function	●
	Testosterone level	●
Protein Expression[4]	Protein levels using western blot analysis	●
Gene Expression[5]	Corpora cavernosa smooth muscle cells, gene up- or down-regulation	●

● = continuous dose; □ = stop dose.
mo = months; bw = body weight.
[1]Male Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose BPA treatments. Animals were examined for effects at 6 months.
[2]Endpoint list: percent of positive area versus total area (smooth muscle area, collagen area, lacunar space area); percent integrated optical density (smooth muscle area, collagen area, lacunar space area).
[3]Endpoint list: basal intracavernosal pressure, intracavernosal pressure following papaverine hydrochloride, drop rate in intracavernosal pressure within next minute following saline infusion (by dynamic infusion cavernosometry); ratio between maximal intracavernosal pressure and mean arterial pressure at the peak of erectile response (by electrical field stimulation); serum testosterone.
[4]Endpoint list: penile shaft collagen (per mg tissue, per µg total protein); calponin 1 (Calp 1); α-smooth muscle actin (αSMA); vascular endothelial growth factor (VEGF); endothelial nitric oxide synthase (eNOS); neuronal nitric oxide synthase (nNOS); inducible nitric oxide synthase (iNOS); nitric oxide synthase 2A (NOS2A) IgG; brain-derived neurotrophic factor (BDNF).
[5]Global gene transcriptional profiles (signatures) were obtained from smooth muscle cell cultures. Only genes up- or down-regulated by at least twofold compared with controls were considered.

Study Summaries

Core Study: NTP (2018)

Methods

There were no measurements taken in the core study that reflect influences of BPA on penile function.

Investigational Study: Gonzalez-Cadavid (Unpublished)

Methods

Electrical field stimulation of the cavernosal nerve (EFS) was performed on animals at the NCTR facility by staff from the Gonzalez-Cadavid laboratory. The cavernosal nerve was exposed and attached to a bipolar platinum electrode. Systemic arterial and intracavernosal pressure (ICP) measurements were obtained by simultaneous femoral artery and cavernosal catheterization, respectively. EFS was applied at increasing voltages up to 10 V and a frequency of 15 Hz for 60 seconds, separated by 1-minute intervals. Arterial and ICP were simultaneously recorded. The ratio between the maximal intracavernosal pressure (MIP) and the mean arterial pressure (MAP) at the peak of erectile response was calculated.

Dynamic Infusion Cavernosometry involved determining basal intracavernosal pressure (ICP) prior to administration of papaverine hydrochloride into the corpora cavernosa. The ICP during tumescence was recorded 5 minutes after the injection. After the ICP decreased below 40 mmHg, saline was infused, increasing infusion rate by 0.05 ml/min every 10 seconds, until the ICP reached 100 mmHg; the infusion rate was then adjusted to maintain a steady ICP level just above 100 mmHg. The "drop rate" was determined by recording the fall in ICP within the next 1 minute after the infusion was stopped.

The animals were terminated; blood was collected; and serum was prepared, snap frozen, and shipped to the Gonzalez-Cadavid laboratory. Testosterone was assayed by a validated LC-MS/MS method.

Aliquots of the skin-denuded penile shafts were fixed in 10% formalin overnight and shipped to the Gonzalez-Cadavid laboratory, where they were processed for paraffin embedded tissue sections. Adjacent tissue sections were used for Masson trichrome staining for collagen and smooth muscle cells. Aliquots of the penile shaft were also embedded for obtaining frozen tissue sections at NCTR that were subjected to Oil Red O staining for detecting fat droplets at the Gonzalez-Cadavid laboratory. Quantitative image analysis was performed by computerized densitometry. For all determinations, only the corpora cavernosa and the tunica albuginea were analyzed in a computerized grid and expressed as a percentage of positive area versus total area. In all cases, the total penile cross section was analyzed per tissue section, with at least three matched sections per animal and eight animals per group.

Penile tissue homogenates were prepared at NCTR and shipped to the Gonzalez-Cadavid laboratory. Supernatant proteins were subjected to western blot analyses by polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and nonspecific binding was blocked. The membranes were incubated with primary antibodies including calponin 1, α-smooth muscle actin, vasoendothelial growth factor, endothelial nitric oxide synthase, neuronal nitric oxide synthase, inducible nitric oxide synthase, nitric oxide synthase 2A IgG, brain-derived neurotrophic factor, and the reference housekeeping protein mouse glyceraldehyde 3-phosphate dehydrogenase. The washed membranes were incubated with monoclonal antibody, followed by a secondary antibody linked to horseradish peroxidase. Immuno-reactive bands were visualized and densitometric analyses of the bands were performed.

For collagen estimation, tissue was homogenized followed by the estimation of hydroxyproline by a modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent.

For smooth muscle cell cultures from the rat penis, fresh penile shaft was excised from two rats from each dose group, and skin and fascia were denuded and then shipped overnight on wet ice pads to the Gonzalez-Cadavid laboratory. Smooth muscle cultures were obtained, expanded to the third or fourth passage, and used for RNA isolation. Global gene transcriptional profiles (signatures) were obtained. Assays were performed in a duplicate set of penile tissue RNAs by the University of California-Los Angeles DNA microarray core, applying the Affymetrix Rat Gene array for 29,215 sequences. Only genes that were up- or down-regulated by at least twofold were considered unless specifically detailed.

Findings

Unpublished data from this study are available at: https://doi.org/10.22427/NTP-DATA-018-00009-0001-000-9.

Chapter 7. Prostate Gland and Urethra

Summarized below are findings of the CLARITY-BPA core study (NTP 2018) and the investigational studies by the laboratory of Gail Prins (Prins et al. 2018) as well as the laboratories of Frederick vom Saal and collaborator William Ricke (Uchtmann et al. 2020) with respect to the potential effects of BPA on the prostate gland and urethra. All individual animal data for these studies are available online at https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA . The study details, including endpoints measured and timing of assessment, are detailed in Table 15. The statistically significant findings from each investigational study and the statistically significant findings from the core study on related endpoints are included in Table 16.

Table 15. Study Details and Summary of Measured Prostate Gland and Urethra Endpoints

Endpoint Category	Specific Measure	Uchtmann et al. (2020)[1]	Prins et al. (2018)[2]	NTP (2018)[3]		Prins et al. (2018)[2]		vom Saal (Unpublished)[1]	NTP (2018)[3]	
		Age at Assessment								
		PND 1	6 mo	1 yr					2 yr	
Gene Expression[4]	Dorsolateral prostaspheres, basal progenitor cell marker	–	●	–	–	–	–	–	–	–
	Dorsolateral prostaspheres, luminal progenitor cell marker	–	●	–	–	–	–	–	–	–
	Dorsolateral prostaspheres, neuroendocrine cell marker	–	●	–	–	–	–	–	–	–
	Dorsal prostate	–	–	–	–	–	□	–	–	–
Protein Expression[5]	Protein levels in prostate using immunohistochemistry	●	–	–	–	–	–	●	–	–
Pathology[6]	Dorsolateral prostate	–	–	●	□	–	–	–	●	□
	Dorsolateral prostaspheres, number of cells and cell size	–	●	–	–	–	–	–	–	–
	Lateral prostate	–	–	–	–	●	□	–	–	–
	Periurethral prostatic ducts[7]	–	–	–	–	●	□	–	–	–
	Ventral prostate	–	–	●	□	●	□	–	●	□
Morphology[8]	Collagen deposition	–	–	–	–	–	–	●	–	–
	Urethra, morphological changes	●	–	–	–	–	–	●	–	–

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.
yr = year; mo = month; PND = postnatal day; bw = body weight.
[1]Male Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose BPA treatments. Animals were sacrificed at PND 1 or 1 yr and examined for effects. The data from 1-yr-old males were not included in the published paper and are not described here.
[2]Male Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 6 mo (for gene expression) or 1 yr (for pathology) and examined for

effects. Prostaspheres were generated from dorsolateral prostate stem cells. Animals from the stop-dose BPA treatments were also sacrificed at PND 90, but the biological samples collected were not analyzed.
[3]Male Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr or 2 yr and examined for effects.
[4]Prins et al. 2018 endpoint list: gene expression in passage 3 prostaspheres with or without exposure to estradiol-17β (E2) for basal progenitor cell markers (*CK5*, *Hoxb13*, *Sox2*, and *Sox9*); luminal progenitor cell markers (*CK8*, *Tbx3*, and *Trop2*); and neuroendocrine cell marker (chromogranin A) [0, 2.5, 25, and 250 µg BPA/kg bw/day only].
Prins (unpublished) endpoint list: whole-genome RNA-seq counts of dorsal prostate. Data are available in CEBS at https://doi.org/10.22427/NTP-DATA-018-00003-0001-000-3.
[5]Uchtmann et al. 2020 endpoint list: Immunohistochemistry was performed using antibodies that focused on the urethral epithelium nearest to the widest part of the urethra as follows: AR (androgen receptor), CYP19A1 (aromatase), CYP11A1 (cytochrome P450), BMP4 (bone morphogenetic protein 4), DKK2 (dickkopf), ESR1 (estrogen receptor 1; also called estrogen receptor alpha), IGF-1 (insulin-like growth factor-1), SF-1 (steroidogenic factor 1; NR5A1), Thbs2 (thrombospondin 2), and SFRP4 (secreted frizzled-related protein 4) [PND 1].
vom Saal (unpublished) endpoint list: AR (androgen receptor) and estrogen receptor alpha (ERα); data available at: https://doi.org/10.22427/NTP-DATA-018-00013-0001-000-4.
[6]NTP 2018 endpoint list: comprehensive assessment of neoplastic and nonneoplastic lesions, including adenoma; adenoma or adenocarcinoma; atrophy; epithelium hyperplasia; fibrosis; infiltration, cellular, lymphocyte mineralization; mucinous cyst; and suppurative inflammation.
Prins et al. 2018 endpoint list: total prostasphere number (spheres > 40 µm size; with or without E2) and prostasphere size [number of spheres size 40–80 µm and size > 80 µm (without E2)] [0, 2.5, 25, and 250 µg BPA/kg bw/day only].
In the prostate: inflammatory cell infiltration (inflammation), reactive epithelial hyperplasia, prostatic intraepithelial neoplasia (PIN lesion), and adenocarcinoma (evaluated in animals with or without testosterone and E2 (T+E2) implants at PND 90) [all doses].
[7]Includes PIN and neoplastic lesions of anterior prostatic ducts and dorsolateral prostatic ducts evaluated in stop-dose rats.
[8]Uchtmann et al. 2020 endpoint list [PND 1]:
Colliculus: colliculus angle ($C_{A°}$); colliculus volume (C_V); and colliculus surface area (C_{SA}).
Cranial region of the urethra: cranial urethral distance (CU_O); cranial urethral length, cranial to caudal (CU_L); cranial urethral lower angle (CU_{LA}); cranial urethral surface area (CU_S); cranial urethral upper angle (CU_{UA}); cranial urethral volume (CU_V); cranial urethral width, and dorsal to ventral (CU_W).
Midway and widest portions of the urethral lumen: colliculus distance (C_D); colliculus size (C_{SA}); luminal area (L_A); luminal perimeter (L_P); urethral area (U_A); urothelium area (UE_A); urothelium thickness (UE_T); urethral perimeter (U_P); and urethral lateral width (U_{LW}).
Prostatic region of the urethra: prostatic urethral surface area (PU_S); prostatic urethral volume (PU_V).
Total urethra: urethral length, cranial to caudal (U_L); urethral volume (U_V); urethral surface area (U_S); and urethral width, dorsal to ventral (U_W).
vom Saal (unpublished) endpoint list: collagen deposition; bladder lumen (width and wall width); bladder volume [measured by computerized tomography (CT) scan and by calipers]; and urethra lumen (width and wall width); data available at: https://doi.org/10.22427/NTP-DATA-018-00013-0001-000-4.

Study Summaries

Core Study: NTP (2018)

Methods

The study included gross and histological examination of dorsolateral and ventral lobes of the prostate (Table 15) at interim (1 year) and terminal sacrifice (2 years). Prostate weights were not collected.

Findings

Regarding nonneoplastic lesions of the prostate, lymphocytic cellular infiltration of the dorsolateral prostate was higher at 1 year in continuous-dose males exposed to BPA at 2.5 µg/kg bw/day (RTE) compared to controls. Similar results for lymphocytic cellular infiltration were observed in a sensitivity analysis that excluded all animals that overlapped with those exposed to 250,000 µg BPA/kg bw/day (CAFE) (Table 16). In contrast, at 2 years, a lower incidence of lymphocytic cellular infiltration was observed in the dorsolateral prostate in continuous-dose males by trend analysis (Poly-3) and by pairwise comparison with controls at

25,000 µg BPA/kg bw/day (Poly-3, JT/SW, and RTE). A lower incidence of lymphocytic cellular infiltration of the ventral prostate at 2 years was observed in continuous-dose males exposed to BPA (Poly-3 [2.5–250 µg/kg bw/day only], RTE [2.5–250 and 25,000 µg/kg bw/day only], JT/SW) and in stop-dose males exposed to 25,000 µg BPA/kg bw/day group (JT/SW and RTE) at 2 years compared to respective control males.

Suppurative inflammation of the dorsolateral prostate of continuous-dose males was greater in all but the 25 µg/kg bw/day BPA dose (RTE and JT/SW [2,500 and 25,000 µg BPA/kg bw/day exposure groups only]) at 1 year. At 2 years, a higher incidence of suppurative inflammation of the dorsolateral prostate was only observed in the 2.5 µg BPA/kg bw/day group of continuous-dose males compared to controls (Poly-3). In the ventral prostate in continuous-dose males, a lower incidence of suppurative inflammation was observed in BPA-exposed males at 1 year by trend analysis (CAFE, JT/SW, and RTE) and by pairwise comparison (CAFE [2.5, 2,500, and 25,000 µg BPA/kg bw/day dose groups only], JT/SW, and RTE), and at 2 years by pairwise comparison (Poly-3 [2.5–2,500 µg BPA/kg bw/day only], RTE [2.5–2,500 µg BPA/kg bw/day only], and JT/SW) relative to respective controls.

Of the remaining nonneoplastic lesions reported in the prostate, the incidence of fibrosis at 2 years in the dorsolateral prostate was lower in stop-dose males exposed to BPA (JT/SW, RTE [2.5–2,500 µg/kg bw/day only], Poly-3 [2.5, 25, and 2,500 µg/kg bw/day only]). A lower incidence of fibrosis was observed in the ventral prostate by pairwise comparison of BPA-exposed continuous-dose males (RTE [2.5, 250, and 25,000 µg/kg bw/day only] and JT/SW [25,000 µg/kg bw/day only]) and stop-dose males (RTE [25,000 µg/kg bw/day only]) at 2 years relative to respective controls. The incidence of mineralization was significantly lower in the ventral lobe of BPA-exposed continuous-dose males at 2 years by trend analysis (Poly-3, JT/SW, and RTE) and by pairwise comparison with control (JT/SW [25–25,000 µg BPA/kg bw/day only] and RTE). Atrophy of the ventral prostate occurred relatively infrequently and was lower than in controls in the 250 µg BPA/kg bw/day group of continuous-dose males at 2 years (RTE). The incidence of epithelial hyperplasia at 2 years was higher in the ventral prostate of the continuous-dose males exposed to 250 µg BPA/kg bw/day (RTE), while it was lower in the 2.5 µg BPA/kg bw/day exposure group in stop-dose males (Poly-3 and RTE) compared to respective control males.

Ethinyl estradiol

The incidence of suppurative inflammation in the ventral prostate of EE2-exposed continuous-dose males was lower at 1 year by trend analysis (CAFE, JT/SW, and RTE) and by pairwise comparison with controls (CAFE [0.05 µg EE2/kg bw/day only], JT/SW, and RTE). In contrast, at terminal sacrifice, a lower incidence of suppurative inflammation (RTE) as well as lower incidences of fibrosis and lymphocytic cellular infiltration (Poly-3 and RTE) were observed in the ventral prostate of 0.05 µg EE2/kg bw/day exposed continuous-dose males relative to controls.

Author conclusions

The authors of the core study reported there were no exposure-related differences in the incidences of neoplasms in exposed males versus controls in the continuous-dose or stop-dose arms at interim or terminal sacrifice. Regarding increases in nonneoplastic lesions, they stated there was higher incidence of lymphocyte infiltration and suppurative inflammation in the

dorsolateral prostate in BPA-exposed continuous-dose males at 1 year. The authors reported that a lower incidence of suppurative inflammation of the ventral prostate was seen in some dose groups of continuous-dose BPA males at 1 and 2 years, but not in stop-dose males. They commented that there was a high background of inflammation in the dorsolateral prostate in the interim and terminal sacrifice animals, which the authors noted has been linked in the literature to increased prolactin levels with aging, although prolactin was not measured in the current study.

Investigational Study: Prins et al. (2018)

Methods

The study was conducted in two experiments that tested whether BPA exposure leads to prostate pathology (experiment 1, continuous-dose males [Set 1] and stop-dose males [Set 2]) and/or sensitizes prostatic susceptibility to hormonally induced carcinogenesis (experiment 1, stop-dose males [Set 3]), and whether chronic low-dose BPA affects prostate epithelial stem and progenitor cells (experiment 2 conducted on stem cells from 6-month-old continuous-dose males). In the first experiment, male rats were taken from the continuous-dose arm (Set 1) and stop-dose arm (Set 2) at 1 year, and the entire prostatic complex, including all prostate lobes and the periurethral prostatic ducts, was removed. Prostates from the continuous-dose arm animals were fixed in 10% formalin, shipped to the Prins laboratory, and processed for histological examination of pathological lesions. In the stop-dose arm, one lobe each of ventral, dorsal, and lateral prostate from the right side of the prostate were collected, snap frozen, and shipped to the Prins laboratory for global transcript analysis by RNA-seq (unpublished data). The remaining accessory sex gland complex (including the left prostatic lobes) were fixed and shipped to Prins laboratory. In order to test for prostatic susceptibility to hormonally induced carcinogenesis, stop-dose males at PND 90 (Set 3) were implanted with Silastic capsules containing testosterone and 17β-estradiol (T+E2) to simulate the hormonal changes associated with aging and prostate carcinogenesis in humans. They were subsequently sacrificed at age 1 year and the prostatic complex was fixed, shipped to the Prins laboratory, processed for histological examination of pathological lesions, and scored according to previously published criteria (Bosland et al. 1995; McCormick et al. 1998), including adenocarcinoma, prostatic intraepithelial neoplasia (PIN; also referred to as dysplasia or atypical hyperplasia), and nonneoplastic lesions (e.g., epithelial hyperplasia, inflammatory cell infiltration [called inflammation], reactive epithelial hyperplasia [located in epithelium near the areas of inflammation]).

In the second experiment, continuous-dose males administered 2.5, 25, or 250 μg BPA/kg bw/day were sacrificed at 6 months, and the prostatic complex was removed and shipped in cold media to the Prins laboratory. The prostatic complex was dissected into dorsolateral and ventral lobes, and the dorsolateral lobes were processed for stem cell isolation using 3D cultures; the prostate complex from two separate males per exposure were pooled for these studies. In 3D cultures, only prostate epithelial stem cells survive while other epithelial cells undergo apoptosis or senescence. These cultured stem cells asymmetrically divide to generate daughter progenitor cells that rapidly divide forming prostaspheres of progenitor cells undergoing commitment to basal and luminal progenitor lineages over 7 days of culture. The cultures were serially passaged every 7 days and the third generation of prostaspheres was cultured for 1 week with or without 1 nM E2 and then processed for spheroid number, spheroid size, and gene expression of prostate stem-progenitor cell and lineage markers by qRT-PCR. The

genes included cytokeratin 5 (*CK5*) to identify basal progenitor cells and cytokeratin 8 (*CK8*) to identify luminal progenitor cells. The lineage markers evaluated for basal progenitor cells were: *Sox2* (sex-determining region Y box 2), *Hoxb13* (homeobox protein Hoxb13), and *Sox9* (sex-determining region Y box 9). The markers for luminal progenitor cells were: *Tbx3* (T-box transcription factor Tbx3) and *Trop2* (tumor-associated calcium signal transducer 2). In addition, expression of the chromogranin gene was used as a marker of neuroendocrine cells. Gene expression data were analyzed using the delta CT method and expression levels of each gene were normalized to housekeeping gene RPL19 levels.

Findings

In the first experiment, BPA exposure did not induce pathology in the prostate or periurethral ducts in either the continuous-dose (Set 1) or stop-dose (Set 2) males at age 1 year. However, in the stop-dose-arm males implanted with T+E2 capsules in adulthood (Set 3), greater PIN severity scores were observed in the lateral lobe of the prostate at 2.5, 250, and 25,000 µg BPA/kg bw/day compared to controls (Table 16). In addition, the multiplicity of adenocarcinoma in the periurethral ducts of the dorsolateral prostate was higher in the 2.5 µg BPA/kg bw/day dose group compared to controls; data from the 25 and 2,500 µg BPA/kg bw/day dose groups of Set 3 males were not evaluated due to each having a small sample size (n = 4). The periurethral ducts of the anterior lobes also exhibited adenocarcinoma in response to the T+E2 implants at age 1 year; however, there were no exposure effects of BPA on this endpoint.

In prostaspheres generated from the stem cells of dorsolateral prostates of 6-month-old continuous-dose males (experiment 2), the total prostasphere number (size > 40 µm, normalized to input epithelial cell number [10,000 cells]) was higher in the 2.5 µg BPA/kg bw/day dose group relative to controls; the authors stated that prostasphere number is an indication of stem cells present in the tissue. The total prostasphere number was also higher in the 250 µg BPA/kg bw/day dose group relative to controls, but the results were not significantly different. In addition, the total number of large prostaspheres (size > 80 µm/10,000 input cells) was greater in the 25 and 250 µg BPA/kg bw/day dose groups relative to controls; the authors stated that the larger size is an indication of a greater progenitor cell proliferation capacity. Exposure to BPA influenced gene expression of the prostasphere cultures with higher expression of genes related to basal progenitor cells and lower expression of luminal progenitor cell markers in the 25 and 250 µg BPA/kg bw/day dose groups.

A similar pattern of effects of BPA in the prostasphere cultures was observed following incubation for 1 week with E2 for total prostaspheres number, size, and gene expression with a few exceptions. For example, following incubation with E2, the 2.5 µg BPA/kg bw/day exposure resulted in higher expressions of luminal progenitor marker of *CK8*, lineage marker *Tbx3*, and basal progenitor cell lineage marker *Hoxb13* compared to controls. The authors suggested that these differences indicate that chronic BPA exposure to 2.5 µg BPA/kg bw/day disturbs lineage commitment if E2 is also present, while chronic BPA doses of 25 or 250 µg BPA/kg bw/day led toward an increased basal progenitor lineage with or without E2.

The data from the prostate tissue of 1-year-old stop-dose males analyzed by RNA-seq are not published in a peer-reviewed publication. Data are available in CEBS at https://doi.org/10.22427/NTP-DATA-018-00003-0001-000-3.

Ethinyl estradiol

Exposure to 0.5 µg EE2/kg bw/day followed by T+E2 implant in adulthood (Set 3) resulted in a higher PIN severity score in continuous-dose males compared to controls. Similar to the results of 25 and 250 µg BPA/kg bw/day dose groups, there were more total prostaspheres and spheres measuring > 80 µm/10,000 input cells in samples generated from EE2-exposed males relative to controls. Gene expression analysis revealed that EE2 exposure resulted in higher expression of basal lineage genes and an inhibition of luminal lineage genes in prostaspheres incubated with or without E2 prior to analysis. The authors reported that the greater number and size of prostaspheres and the higher expression of basal lineage gene in the EE2 group suggested that BPA is working through an estrogen-dependent mechanism.

Author conclusions

The authors concluded that the results supported their hypothesis that developmental BPA exposure sensitizes the prostate to later-life E2-induced carcinogenesis. They stated that the shift in severity of lesions from low-grade to high-grade PIN among BPA- or EE2-exposed animals relative to controls with only low-grade PIN lesions (following exposure to T+E2 capsules in adulthood) is similar to the shift to high-grade PIN in humans, the immediate precursor lesion for prostate cancer. Also, at the lowest dose (2.5 µg BPA/kg bw/day), there was a significant increase in adenocarcinoma multiplicity in the dorsolateral prostate ducts, which the authors stated parallels the core study reports of higher lymphocytic infiltration of the dorsolateral prostate at 1 year, which was not seen at higher BPA doses. The authors hypothesized that BPA may increase cancer risk by increasing prostatic stem cell numbers as seen at the lower dose of BPA (2.5 µg BPA/kg bw/day) or by increasing progenitor cell proliferation and altering their lineage commitment toward more basal-cell lineages in the 25 and 250 µg BPA/kg bw/day dosed groups, which may increase the likelihood of tumor initiation by secondary exposures.

Investigational Study: Uchtmann et al. (2020)

Methods

This study from the vom Saal and Ricke laboratories evaluated the development of the urogenital tracts in male rats at PND 1 (Uchtmann et al. 2020) and 1 year old (vom Saal, unpublished; available in CEBS). On PND 1, pup body weights were measured, and pups were sacrificed. The hind end of the male pups was fixed in 10% formalin and shipped to the vom Saal laboratory at the University of Missouri-Columbia. The entire male lower urogenital tract (lower UGT [including the urogenital sinus], bladder, and associated sex glands) were collected, embedded, and sectioned at 5 µm to create a tissue array. Every third slide was then stained for basal epithelial cell nuclear marker p63 to create a 3D reconstruction. Several morphometric measurements were made of the UGT following identification of the widest portion and the midway portion of the urethral lumen. The measurements included: urethral area (U_A), urethral perimeter (U_P), luminal area (L_A), luminal perimeter (L_P), urothelium area (UE_A), urothelium thickness (UE_T), and urethral lateral width (U_{LW}). The colliculus distance (C_D), measured from the lowest point of the colliculus to the lowest point of the urethra, was used as a proxy for the size of the colliculus (C_S); C_S was calculated as $1/C_D$ in µm × 1,000.

Other morphometric measurements of the total urethra included: urethral volume (U_V), urethral surface area (U_S), dorsal to ventral urethral width (U_W), and caudal to cranial urethral length (U_L). For the prostatic region of the urethra, the volume (PU_V) and surface area (PU_S) were

measured. For the urethra associated with the colliculus, the volume (C_V) and surface area were measured as well as the colliculus angle (C_A, which is the angle of the colliculus in relation to the cranial urethra). Morphometric measurements of the cranial region of the urethra were made, including: volume (CU_V), surface area (CU_S), distance (CU_D, which is the distance from the most cranial point evaluated to where the anterior prostate emerged), dorsal to ventral width (CU_W), caudal to cranial length (CU_L), lower angle (CU_{LA}, which is the angle made between the prostatic and cranial urethra on the caudal end), and the upper angle (CU_{UA}, which is the angle made between the prostatic and cranial urethra on the cranial end). To evaluate estrogen-regulated factors and prostatic proteins, immunohistochemistry was performed using 10 different antibodies that focused on the urethral epithelium nearest to the widest part of the urethra: AR (androgen receptor), CYP19A1 (aromatase), CYP11A1 (cytochrome P450), BMP4 (bone morphogenetic protein 4), DKK2 (dickkopf), ESR1 (estrogen receptor 1), IGF-1 (insulin-like growth factor-1), SF-1 (steroidogenic factor 1; NR5A1), Thbs2 (thrombospondin 2), and SFRP4 (secreted frizzled-related protein 4). Following decoding of the data, only animals within a body weight cutoff range of 5–8.1 g were used in the statistical analyses to control for possible differences in stage of development. This resulted in a sample size of 5–6 pups per exposure group. The data were analyzed by ANOVA followed by Fisher's Least Significant Difference. The goodness of fit for nonmonotonicity versus linear response was conducted using R statistical software.

Similarly, at age 1 year, the male rats in the continuous-dose arm were weighed, euthanized, and necropsied. Their urogenital tracts (UGT) were collected, fixed, and shipped to the vom Saal laboratory. The prostatic urethra was embedded and serially sectioned to create a tissue array. Every tenth slide was stained with hematoxylin and eosin for 3D reconstruction. Immunohistochemistry was performed using antibodies for androgen receptor and estrogen receptor, respectively. Finally, slides nearest to the middle of the prostatic urethra were stained with picrosirius red to identify collagen fibers.

Findings

On PND 1, body weight was higher in the 2.5, 25, and 250 µg BPA/kg bw/day dose groups than in controls (body weight data not included in Table 16). The addition of a quadratic term in the R-statistic revealed a better fit for a nonmonotonic relationship between BPA exposure and body weight at PND 1 compared to the linear analysis. Maternal body weight was also greater in the 25 and 250 µg BPA/kg bw/day groups and litter size was not a factor.

Exposure to BPA altered the development of the colliculus (also called the verumontanum), which is derived from the estrogen-sensitive remnant of presumptive cranial vaginal tissue from the fused caudal part of the Mullerian ducts and is associated with the development of the dorsal and lateral prostate in rats. The angle of the colliculus (C_A), which is the angle of the colliculus in relation to the cranial urethra, was lower at BPA doses of 2.5, 25, and 250 µg/kg bw/day (Table 16). The colliculus size (C_S), measured on the widest luminal section of the UGS, was greater in the 250 µg BPA/kg bw/day dose group than in controls due to a lower colliculus distance (C_D). Exposure to BPA narrowed the urethra lumen as evidenced by a lower dorsal-ventral width of the entire urethra (U_W) at the 25 µg BPA/kg bw/day dose and a lower cranial-caudal length of the entire urethra (U_L) at the 2.5 µg BPA/kg bw/day dose. In particular, the cranial urethra had a smaller width (CU_W) at the 25 µg BPA/kg bw/day dose and a smaller length (CU_L) at the 2.5 µg BPA/kg bw/day dose. In addition, the luminal perimeter was lower in the

250 µg BPA/kg bw/day dose group. Finally, the urothelium thickness was lower at the widest section (widest UE_T) in the 25, 250 and 2,500 µg BPA/kg bw/day dose groups than in the controls, as well as at the middle section (midway UE_T) in the 250 µg BPA/kg bw/day dose group. Regarding immunostaining, all antibodies for estrogen and prostatic pathways resulted in staining patterns consistent with the expected localization of each protein in the urogenital sinus of PND 1 control males. The authors stated, "Quantitative assessment of all proteins was inconclusive due to high variability within and between the treatment groups" (Uchtmann et al. 2020).

Ethinyl estradiol

Similar to the BPA-exposed males, exposure to EE2 was associated with increased body weight at PND 1. The angle of the colliculus (C_A) was lower than the controls in the 0.05 µg EE2/kg bw/day dose group. In the widest luminal section of the UGS, colliculus distance (C_D) was lower at 0.5 µg EE2/kg bw/day, which resulted in a greater colliculus size (C_S) than controls at the same EE2 dose. The dorsal-ventral width of the entire urethral width (U_W), cranial-caudal urethral length (U_L), and the luminal perimeter (L_P) of the urethra were smaller in the males treated with 0.05 µg EE2/kg bw/day relative to controls.

The data from 1-year-old males were not included in the paper by Uchtmann et al. (2020); however, the unanalyzed data from these studies are available at: https://doi.org/10.22427/NTP-DATA-018-00013-0001-000-4.

Author conclusions

The vom Saal laboratory concluded that the morphometric analysis of 3D reconstructions of the rat urogenital sinus (UGS) on PND 1 "revealed several significant or trending changes that were consistent with prior published findings, including changes in colliculus angle and an increase in colliculus size, a decrease in urethral lumen length and width due to low, but not high doses of BPA, similar to EE2. [The reduction in colliculus size is a known outcome of elevated fetal estrogen exposure (Glenister 1962; McLachlan et al. 1975).] These changes all indicate that the urethra is smaller, associated with changes in colliculus shape, when exposed to low doses of BPA and EE2" (Uchtmann et al. 2020). The authors stated that "…these data suggest that BPA at lower doses (physiologically relevant), but not the highest dose, mediates its effects on the fetal UGS via estrogenic pathways." The authors also reported increased body weight at birth at 2.5, 25, and 250 µg BPA/kg bw/day as well as both doses of EE2 which they hypothesized could be attributed to NCTR Sprague Dawley rats being "…bred for rapid growth and susceptibility to obesity, which may be exacerbated by exposure to low doses of BPA and EE2 during fetal life"(Uchtmann et al. 2020). The authors hypothesized that the observed decrease in urothelial thickness in animals exposed to BPA (25–2,500 µg/kg bw/day), but not EE2, may represent a nonestrogenic effect of BPA.

Table 16. Summary of Statistically Significant Prostate Gland and Urethra Endpoints

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
CD, PND 1	**Morphology**	**Colliculus angle (C_A)** mean ± SE	–	25.1 ± 1.3	21.2 ± 1.1*	21.3 ± 0.7*	20.7 ± 1**	23 ± 0.9	22 ± 1.8*	Uchtmann et al., 2020
		Cranial urethral length (CU_L) mean ± SE, µM	–	103.3 ± 6.1	77.1 ± 10.8**	97.5 ± 5.2	86.1 ± 5.6	99.1 ± 6	97.5 ± 4.2	Uchtmann et al., 2020
		Cranial urethral width (CU_W) mean ± SE, µM	–	29.3 ± 4.3	33.1 ± 3.9	19.7 ± 1.8*	28.4 ± 3.5	31.1 ± 2.1	26.8 ± 3.8	Uchtmann et al., 2020
		Urethral width (U_W) mean ± SE, µM	–	74.4 ± 5.6	70.8 ± 9	50.8 ± 4.8*	64.4 ± 6.7	64.5 ± 5.4	60.8 ± 7.6	Uchtmann et al., 2020
		Widest colliculus distance (widest C_D) mean ± SE, µM	–	42.3 ± 2.3	37.2 ± 5.4	35.9 ± 1.6	31.6 ± 4.9*	40.3 ± 4.8	37.4 ± 2.6	Uchtmann et al., 2020
		Widest colliculus size (widest C_S) mean ± SE, µM	–	24 ± 1.3	29.3 ± 4.2	28.1 ± 1.2	38.8 ± 9.9*	26.5 ± 3.5	27.5 ± 2.2	Uchtmann et al., 2020
		Widest luminal perimeter (widest L_P) mean ± SE, µM × 10^2	–	4 ± 0.3	3.5 ± 0.2	3.6 ± 0.2	3.4 ± 0.1*	4.1 ± 0.2	3.6 ± 0.3	Uchtmann et al., 2020
		Widest urothelium thickness (widest UE_T) mean ± SE, µM	–	5.2 ± 0.4	4.5 ± 0.5	4.2 ± 0.4*	4 ± 0.3*	4.2 ± 0.1*	4.6 ± 0.3	Uchtmann et al., 2020
CD, 6 mo	**Gene Expression[4,5]**	**BP *CK5* expression (no E2)** mean ± SE, (fold change) [n]	–	1.0 ± 0.10 [4]	1.0 ± 0.24 [5]	2.6 ± 0.43* [3]	2.0 ± 0.16 [5]	–	–	Prins et al., 2018
		BP *Hoxb13* expression (with E2) mean ± SE, (fold change) [n]	–	1.2 ± 0.26 [4]	2.7 ± 0.37* [5]	1.6 ± 0.34 [3]	2.9 ± 0.48** [5]	–	–	Prins et al., 2018
		BP *Hoxb13* expression (no E2) mean ± SE, (fold change) [n]	–	1.0 ± 0.18 [4]	1.6 ± 0.40 [5]	3.1 ± 0.56* [3]	2.5 ± 0.38 [5]	–	–	Prins et al., 2018
		BP *Sox2* expression (no E2) mean ± SE, (fold change) [n]	–	1.0 ± 0.076 [4]	1.2 ± 0.33 [5]	3.6 ± 0.72** [3]	2.4 ± 0.22 [5]	–	–	Prins et al., 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
		LP *CK8* expression (with E2) mean ± SE, (fold change) [n]	–	0.79 ± 0.038 [4]	1.3 ± 0.039 **** [5]	0.065 ± 0.015 **** [3]	0.094 ± 0.030 **** [5]	–	–	Prins et al., 2018
		LP *CK8* expression (no E2) mean ± SE, (fold change) [n]	–	1 ± 0.094 [4]	1.3 ± 0.19 [5]	0.088 ± 0.022 **** [3]	0.10 ± 0.032 **** [5]	–	–	Prins et al., 2018
		LP *Tbx3* expression (with E2) mean ± SE, (fold change) [n]	–	0.62 ± 0.071 [4]	1.2 ± 0.24* [5]	0.20 ± 0.041 [3]	0.33± 0.030 [5]	–	–	Prins et al., 2018
		LP *Trop2* expression (no E2) mean ± SE, (fold change) [n]	–	1.0 ± 0.06 [4]	0.86 ± 0.21 [5]	0.40 ± 0.051* [3]	0.34 ± 0.042** [5]	–	–	Prins et al., 2018
	Pathology[6]	**Total prostasphere number > 40 µm (with E2)** mean ± SE, (/10,000 cells) [n]	–	231 ± 8.2 [4]	510 ± 53.9 [5]***	325 ± 128 [3]	565 ± 177 [5]	–	–	Prins et al., 2018
		Total prostasphere number > 40 µm^2 (no E2) mean ± SE, (/10,000 cells) [n]	–	210 ± 19.4 [4]	401 ± 42* [5]	243 ± 94.4 [3]	438 ± 140 [5]	–	–	Prins et al., 2018
		Total prostasphere number > 80 µm^2 (no E2) mean ± SE, (/10,000 cells) [n]	–	9.8 ± 1.9 [4]	11.7 ± 1.3 [5]	161 ± 61.4** [3]	51.5 ± 10.8* [5]	–	–	Prins et al., 2018
CD, 1 yr	**Pathology[7]**	**Infiltration, cellular, lymphocyte (dorsolateral lobe)** Incidence[8]/n, % incidence (severity profile)	NS	4/22, 18% (4|0|0|0)	10/22, 46%$^{\#9,\wedge 10}$ (9|1|0|0)	5/20, 25% (5|0|0|0)	6/24, 25% (5|1|0|0)	5/20, 25% (5|0|0|0)	7/22, 32% (5|2|0|0)	NTP, 2018
		Suppurative inflammation (dorsolateral lobe) incidence/n, % incidence (severity profile)	NS	18/22, 82% (11|7|0|0)	20/22, 91%$^{\#9,\wedge}$ (6|14|0|0)	18/20, 90% (9|9|0|0)	22/24, 92%$^{\wedge}$ (9|12|1|0)	18/20, 90%$^{\#,\wedge}$ (6|10|2|0)	19/22, 86%$^{\#}$ (7|11|1|0)	NTP, 2018
		Suppurative inflammation (ventral lobe) incidence/n, % incidence (severity profile)	↓$^{**,\#\#,\wedge\wedge}$	10/22, 45% (9|1|0|0)	3/22, 14%$^{N*,\#,\wedge\wedge}$ (3|0|0|0)	4/20, 20%$^{N\#,\wedge}$ (2|2|0|0)	5/24, 21%$^{N\#,\wedge}$ (4|1|0|0)	3/20, 15%$^{N*,\#,\wedge\wedge}$ (3|0|0|0)	1/22, 5%$^{N**,\#\#\#,\wedge\wedge\wedge}$ (1|0|0|0)	NTP, 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
SD, 1 yr	**Pathology**	**Adenocarcinoma (with T+E2 implants at PND 90)**[11] incidence/n, % incidence (tumor multiplicity)	–	8/16, 50% (1.1 ± 0.1)	3/6, 50%** (5.3 ± 1.5)	1/4, 25% (–)12	4/12, 25% (1.8 ± 0.5)	1/4, 25% (–)11	3/9, 34% (2.0 ± 0.6)	Prins et al., 2018
		Prostatic intraepithelial neoplasia (with T+E2 implants at PND 90)[13] incidence/n, % incidence (severity score)	–	14/16, 88% (1.3 ± 0.2)	6/6, 100%** (3.0 ± 0.4)	2/4, 50% (–)12	11/12, 92%* (2.3 ± 0.3)	4/4, 100% (–)12	9/9, 100%* (2.4 ± 0.4)	Prins et al., 2018
CD, 2 yr	**Pathology**[7]	**Atrophy (ventral lobe)** incidence/n, % incidence (severity profile)	NS	4/50, 8% (0|0|3|1)	2/48, 4% (0|0|1|1)	2/48, 4% (0|2|0|0)	0/49, 0%N^ (–)	5/49, 10% (0|1|4|0)	5/46, 11% (0|0|4|1)	NTP, 2018
		Epithelium hyperplasia (ventral lobe) incidence/n, % incidence (severity profile)	NS	10/50, 20% (1|6|3|0)	12/48, 25% (2|6|3|1)	10/48, 21% (2|4|4|0)	18/49, 37%#9,^ (1|13|3|1)	12/49, 24% (3|6|3|0)	8/46, 17% (2|2|2|2)	NTP, 2018
		Fibrosis (ventral lobe) incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	NS	15/50, 30% 15/36.0, 42% (0|7|3|5)	4/48, 8%N##,^^ 4/34.6, 12%N** (2|1|0|1)	6/48, 13%N##,^^ 6/34.2, 18%N* (3|2|1|0)	8/49, 16%N##,^ 8/34.6, 23% (2|3|0|3)	4/49, 8%N###,^^ 4/33.7, 12%N** (1|1|0|2)	11/46, 24%N# 11/31.6, 35% (1|5|2|3)	NTP, 2018
		Infiltration, cellular, lymphocyte (dorsolateral lobe) incidence/n, % incidence Poly-3 incidence/adjusted n, % incidence (severity profile)	↓*	33/50, 66% 33/40.4, 82% (19|11|2|1)	26/48, 54% 26/38.2, 68% (18|7|1|0)	27/48, 56% 27/38.7, 70% (21|5|1|0)	27/50, 54% 27/39.6, 68% (16|6|3|2)	27/50, 54% 27/38.7, 70% (18|8|0|1)	20/46, 43%N#,^ 20/33.0, 61%N* (10|7|1|2)	NTP, 2018
		Infiltration, cellular, lymphocyte (ventral lobe) incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	NS	25/50, 50% 25/40.8, 61% (12|5|5|3)	14/48, 29%N##,^^ 14/38.2, 37%N* (9|4|1|0)	15/48, 31%N##,^^ 15/39.1, 38%N* (14|1|0|0)	15/49, 31%N##,^ 15/39.4, 38%N* (9|4|0|2)	20/49, 41%N# 20/39.0, 51% (15|3|1|1)	15/46, 33%N#,^ 15/33.3, 45% (9|3|1|2)	NTP, 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
		Mineralization (ventral lobe) incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	↓*,#,^	4/50, 8% 4/34.0, 12% (0|0|3|1))	1/48, 2%N^ 1/34.0, 3% (1|0|0|0)	0/48, 0%N#,^^ 0/32.3, 0% (–)	1/49, 2%N#,^ 1/32.9, 3% (1|0|0|0)	1/49, 2%N#,^ 1/33.1, 3% (0|1|0|0)	0/46, 0%N##,^^ 0/28.5, 0% (–)	NTP, 2018
		Suppurative inflammation (dorsolateral lobe) Poly-3 incidence/adjusted n, % incidence (severity profile)	NS	41/45.6, 90% (8|26|4|3)	46/46.2, 100%* (10|26|9|1)	47/48, 98% (9|30|8|0)	45/47.7, 94% (17|24|2|2)	43/46.5, 92% (8|28|6|1)	41/43.3, 95% (12|20|6|3)	NTP, 2018
		Suppurative inflammation (ventral lobe) incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	NS	16/50, 32% 16/38.1, 42% (7|2|2|5)	5/48, 10%N##,^^ 5/34.6, 14%N** (3|2|0|0)	5/48, 10%N##,^^ 5/34.6, 14%N** (3|2|0|0)	6/49, 12%N##,^^ 6/35.3, 17%N* (3|1|0|2)	5/49, 10%N##,^^ 5/34.9, 14%N** (1|2|1|1)	11/46, 24%N## 11/31.9, 34% (5|2|1|3)	NTP, 2018
SD, 2 yr	**Pathology**[7]	**Epithelium hyperplasia (ventral lobe)** incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	NS	17/48, 35% 17/38.9, 44% (0|12|3|2)	7/47, 15%N##9,^^ 7/32.9, 21%N* (2|3|1|1)	17/47, 36% 17/36.0, 47% (4|8|3|2)	16/50, 32% 16/35.7, 45% (4|9|1|2)	14/49, 29% 14/37.4, 37% (1|13|0|0)	13/45, 29% 13/30.2, 43% (1|10|0|2)	NTP, 2018
		Fibrosis (dorsolateral lobe) incidence/n, % incidence (severity profile)	↓#,^	15/46, 33% (1|5|6|3)	8/48, 17%N#9,^ (1|3|1|3)	11/48, 23% (3|4|1|3)	8/50, 16%N#9,^ (1|4|0|3)	12/49, 24% (2|6|4|0)	6/45, 13%N#,^ (0|4|0|2)	NTP, 2018
		Fibrosis (ventral lobe) incidence/n, % incidence (severity profile)	NS	17/48, 35% (2|9|3|3)	10/47, 21% (0|7|0|3)	12/47, 26% (3|5|0|4)	10/50, 20% (1|3|3|3)	12/49, 25% (3|7|2|0)	8/45, 18%N^ (0|1|4|3)	NTP, 2018
		Infiltration, cellular, lymphocyte (dorsolateral lobe) incidence/n, % incidence (severity profile)	NS	31/46, 67% (17|11|1|2)	30/48, 62% (20|6|1|3)	28/48, 58% (15|9|2|2)	27/50, 54% (16|8|1|2)	35/49, 71% (20|14|1|0)	22/45, 49%N#,^ (14|7|0|1)	NTP, 2018
		Coagulating gland atrophy incidence/n, % incidence (severity profile)	NS	1/45, 2% (0|0|1|0)	1/45, 2% (0|0|1|0)	4/44, 9%^ (0|1|1|2)	1/50, 2% (0|1|0|0)	2/48, 4% (0|0|2|0)	2/44, 5% (0|0|1|1)	NTP, 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
		Seminal vesicle lumen dilatation Poly-3 incidence/ adjusted n, % incidence incidence/n, % incidence (severity profile)	NS	6/39, 15% 6/31.6, 19% (0|1|4|1)	4/43, 9% 4/30.6, 13% (0|1|2|1)	2/41, 5%$^{N\wedge}$ 2/30.5, 7% (0|1|0|1)	3/47, 6% 3/30.1, 10% (0|1|1|1)	1/48, 2%$^{N\#\#9,\wedge}$ 1/35.3, 3%^{N*} (0|1|0|0)	4/42, 10% 4/27.0, 15% (0|2|1|1)	NTP, 2018

CD = continuous dose; SD = stop dose; BP = basal progenitor; LP = luminal progenitor; E2 = estradiol-17b; T+E2 = testosterone and estradiol; hyphen (–) = not evaluated.
[1]Shading indicates statistical significance. Statistical tests varied by reference (see subsequent footnotes).
[2]↑ = positive trend, ↓ = negative trend, NS = no significant trend.
[3]Statistical analyses by one-way ANOVA followed by LSmeans test (*, statistically significant at $p < 0.05$; **, statistically significant at $p < 0.01$).
[4]Gene expression data were analyzed using the $^{\wedge}(-\Delta\Delta)C_t$ method and expression levels of each gene were normalized to housekeeping gene RPL19 levels.
[5]Statistical analyses by one-way ANOVA followed by Tukey-Kramer multiple comparison test (*, statistically significant at $p < 0.05$; **, statistically significant at $p < 0.01$; ***, statistically significant at $p < 0.005$; ****, statistically significant at $p < 0.001$).
[6]Statistical analyses by one-way ANOVA followed by Games-Howell multiple comparison test (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$; ***, statistically significant at $p \leq 0.001$).
[7]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected.
Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.
Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.
Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests. All pairwise tests were one-sided and not corrected for multiple comparisons.
Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs.
Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; ***, ###, ^^^, statistically significant at $p \leq 0.001$; N superscript, negative trend, or negative relative to control.
[8]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.
[9]SW test was nonsignificant due to the assumption of monotonicity; thus, this JT/SW data analysis result was not summarized in the CLARITY-BPA integrated report.
[10]In the sensitivity analysis that excluded all animals that overlapped with animals treated with 250,000 µg BPA/kg bw/day (see Statistical Methods), there was a significant CAFE test ($p = 0.025$) for the pairwise comparison of the 2.5 µg BPA/kg bw/day group to the vehicle control group (lymphocyte cellular infiltration, 9/16 [56%] versus 3/17 [18%]).
[11]Statistical analysis by Fisher's exact test with Bonferroni correction.
[12]Not statistically evaluated due to severe underpowering and nonnormality of data.
[13]Statistical analysis by ANOVA with Dunnett's posttest. Only the severity scores (not incidences) for the 2.5, 250, and 25,000 µg/kg bw/day dose groups were significant compared with the control.

Chapter 8. Testis and Epididymis

Findings of the CLARITY-BPA core study (NTP 2018) and the investigational study by the laboratory of Kim Boekelheide (Dere et al. 2018) with respect to the potential effects of BPA on the testis and epididymis are summarized below. All individual animal data for these studies are available online (https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA). The study details, including endpoints measured and timing of assessment, are detailed in Table 17. The statistically significant findings from the investigational study and the statistically significant findings from the core study on related endpoints are included in Table 18.

Table 17. Study Details and Summary of Measured Testis and Epididymis Endpoints

Endpoint Category	Specific Measure	Dere et al. (2018)[1]	NTP (2018)[2]			
		Age at Assessment				
		90 d	1 yr		2 yr	
Organ Weight[3]	Epididymis weight	●	●	□	–	–
	Seminal vesicle		●	□	–	–
	Testis weight	●	●	□	–	–
Pathology[4]	Coagulating gland	–	●	□	●	□
	Epididymis	–	●	□	●	□
	Seminal vesicle	–	●	□	●	□
	Testis	●	●	□	●	□
Male Reproductive Function[5]	Cauda epididymis, sperm DNA methylation levels	●	–	–	–	–
	Cauda epididymis, sperm mRNA levels	●	–	–	–	–
	Cauda epididymis, sperm morphology	–	●	□	–	–
	Cauda epididymis, sperm count	–	●	□	–	–
	Cauda epididymis, sperm motility	–	●	□	–	–
	Testis, homogenization-resistant spermatid head counts	●	●	□	–	–
	Testis, apoptotic germ cells	●	–	–	–	–
	Testis, retained spermatid heads	●	–	–	–	–

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.
yr = year; d = day; PND = postnatal day.
[1]Male Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, 25,000 or 250,000 µg/kg bw/day using continuous-dose BPA treatment. Animals were sacrificed at PND 90 and examined for effects. Animals were sacrificed also at 1 yr, but the biological samples collected were not analyzed.
[2]Male Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr or 2 yr and examined for effects.
[3]NTP 2018 endpoint list: epididymis weight (paired): absolute, relative to body weight, relative to brain weight; seminal vesicle weight (paired): absolute, relative to body weight, relative to brain weight; testis weight (paired): absolute, relative to body weight, relative to brain weight.
Dere et al. 2018 endpoint list: epididymis weight: absolute; testis weight: absolute.
[4]NTP 2018 endpoint list: comprehensive assessment of neoplastic and nonneoplastic lesions (e.g., epididymis: hypospermia, exfoliated germ cells, lymphocyte infiltration, polyarteritis; testis: polyarteritis, seminiferous tubule degeneration).
Dere et al. 2018 endpoint list: testis, altered germ cell apoptosis: count of TUNEL-positive cell nuclei.
[5]NTP 2018 endpoint list: cauda epididymis: sperm count (head, tail, and head and tail combined), sperm motility (% motile), sperm morphology (% abnormal per sperm head, tail, and head and tail combined); testis: spermatid head counts.
Dere et al. 2018 endpoint list: cauda epididymis: sperm DNA methylation levels, sperm mRNA levels; testis: number of retained spermatid head count per tubule (assessment of disrupted spermiation), sperm count (number of homogenization-resistant spermatid heads per testes), and apoptotic germ cells (% tubules with >3 TUNEL-positive cell nuclei).

Study Summaries

Core Study: NTP (2018)

Methods

There were several endpoints evaluated in the core study related to the testis and epididymis (Table 17). These included testis and epididymis weights, sperm evaluations at 1 year, and histopathology at 1 year and 2 years for both stop- and continuous-dose groups.

Findings

In the epididymis of stop-dose males, the incidence of exfoliated germ cells was increased compared to controls in the 2.5 μg BPA/kg bw/day dose group at 1 year (RTE), and a lower incidence of epididymis hypospermia was observed in the 250 μg BPA/kg bw/day dose group compared to controls at 2 years (RTE) (Table 18).

Also in the epididymis, in continuous-dose BPA males at 1 year, there were positive trends (CAFE, RTE, JT/SW) for exfoliated germ cells and lymphocyte infiltration. With both lesions, the incidence in the 25,000 μg BPA/kg bw/day dose group was markedly increased, and the positive trend for exfoliated germ cells was due to the elevated incidence in the 25,000 μg BPA/kg bw/day dose group. There was an increase in polyarteritis of the epididymis in 2,500 μg BPA/kg bw/day continuous-dose males at 2 years (JT/SW and RTE).

In the testis, in continuous-dose males at 2 years, there was a lower incidence of seminiferous tubule degeneration in the 2.5 μg BPA/kg bw/day group than in controls (Poly-3). Polyarteritis of the testis occurred with an increasing dose-related trend (Poly-3, JT/SW, and RTE) and was increased in the 2,500 μg BPA/kg bw/day stop-dose group compared to controls (Poly-3, JT/SW, and RTE).

In sperm assessments, there were no effects of BPA exposures on testicular spermatid head counts, caudal sperm counts, and caudal sperm motility and morphology in any dose group at the 1-year evaluation.

Ethinyl estradiol

In the continuous-dose animals exposed to EE2, there were no changes reported for testis weights, histopathologic findings, or sperm parameters when compared to controls. In the epididymis, lymphocytic cellular infiltration occurred with a positive trend and was increased compared to controls in the 0.5 μg EE2/kg bw/day group at 1 year (JT/SW and RTE) and 2 years (RTE).

Author conclusions

The authors concluded that BPA had no biologically meaningful effects on the testis and epididymis.

Investigational Study: Dere et al. (2018)

Methods

Males were taken from the continuous-dose arm at PND 90. Left testes were fixed in 10% formalin, and the right testes were snap frozen and shipped to the Boekelheide lab where they

were processed for histopathological evaluation, homogenization-resistant sperm head counts, retained spermatid heads, and apoptosis measures (Table 17). Sperm were isolated from the epididymis at the time of necropsy and processed for RNA and DNA analysis. An additional higher dose group was included in this study, 250,000 µg BPA/kg bw/day; these animals (n = 20/group; 2 pups/litter) originated from a separate cohort and load of animals and had a separate concurrent vehicle control group.

Findings

Decreased testis and epididymis weights were observed in the high-dose (250,000 µg BPA/kg bw/day) group, consistent with lower body weights (Table 18). Quantification of retained spermatid heads in the basal compartment of the seminiferous tubules provided an assessment of disrupted spermiation, while sperm production was evaluated by quantifying homogenization-resistant spermatid heads in testis homogenates. TUNEL-positive cell nuclei provided a measure of apoptotic germ cells. There were no effects of BPA exposure at any dose on these endpoints. There were no groups of animals that received EE2 as part of the testis/epididymis evaluations in the Dere et al. (2018) study.

Sperm transcriptomic analysis was performed to evaluate mRNA content across the dose levels. Increases in altered probes were observed at the 250 and 2,500 µg BPA/kg bw/day dose levels, but there was no consistency in individually altered probes using a Monte Carlo-based analysis, which suggested that BPA exposure did not "robustly" alter sperm mRNA levels. Global changes in CpG methylation were assessed using a reduced representation bisulfite sequencing method. The greatest number of differentially methylated regions (DMRs) was found in the 250 µg BPA/kg bw/day dose group, and the fewest at 2.5 µg BPA/kg bw/day, with the number of DMRs at 2.5 µg BPA/kg bw/day being similar to the DMRs between two vehicle control groups (core study controls versus controls run concurrent with the 250,000 µg BPA/kg bw/day dose group).

The initial analysis of mRNA abundance and DNA methylation in sperm following continuous BPA exposure from early gestation suggested a possible nonmonotonic dose-response, with the middle dose of 250 µg BPA/kg bw/day eliciting the greatest number of dysregulated genes and DMRs.

Author conclusions

The authors concluded, "Taken at face value, these results agree with previous studies reporting nonmonotonic dose-relationships with BPA. However, using additional modeling approaches, we concluded that these responses lacked robustness and reproducibility, and should not be considered BPA-elicited responses. This conclusion is based on our analysis of: (1) iterative (Monte Carlo) analyses with randomly selected subsets of samples, which failed to consistently identify dysregulated transcripts or a common set of DMRs, (2) a relatively high background frequency of DMRs detected in an analysis comparing the two groups of vehicle controls, and (3) dose-response modeling of BPA-elicited DMRs, which poorly fit the DNA methylation data of both linear and nonlinear dose-response models. The collective findings from these independent analyses strongly suggest that BPA did not alter sperm DNA methylation" (Dere et al. 2018).

Table 18. Summary of Statistically Significant Testis and Epididymis Endpoints

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	250,000	
CD, 90 d	**Organ Weight[3]**	**Epididymis weight: absolute** mean ± SD, (mg) [n]	–	579.9 ± 44.7 [10]	–	–	–	–	–	528.8 ± 32.9 * [9]	Dere et al., 2018
		Testis weight: absolute mean ± SD, (g) [n]	–	1.794 ± 0.092 [10]	–	–	–	–	–	1.554 ± 0.115* [9]	Dere et al., 2018
CD, 1 yr	**Pathology[4]**	**Epididymis, exfoliated germ cells** incidence[5]/n, %incidence (severity profile)	↑*, #,^	1/22, 4% (1|0|0|0)	1/22, 4% (1|0|0|0)	1/20, 5% (0|1|0|0)	1/24, 4% (1|0|0|0)	0/20, 0% (NA)	6/22, 27%*,##,^^ (6|0|0|0)	–	NTP, 2018
		Epididymis, lymphocyte infiltration incidence/n, %incidence (severity profile)	↑*, #,^	0/22, 0% (NA)	1/22, 4% (0|1|0|0)	3/20, 15%#7 (3|0|0|0)	2/24, 8% (2|0|0|0)	0/20, 0% (NA)	5/22, 23%*,##,^^ (5|0|0|0)	–	NTP, 2018
SD, 1 yr	**Pathology[4]**	**Epididymis, exfoliated germ cells** incidence/n, %incidence (severity profile)	NS	0/20, 0% (NA)	3/20, 15%^ (1|2|0|0)	1/20, 5% (0|1|0|0)	2/19, 10% (2|0|0|0)	1/20, 5% (0|1|0|0)	1/22, 4% (1|0|0|0)	–	NTP, 2018
CD, 2 yr	**Pathology[4]**	**Epididymis polyarteritis** incidence/n, %incidence (severity profile)	NS	0/49, 0% (NA)	1/48, 2% (0|0|1|0)	1/48, 2% (0|1|0|0)	1/50, 2% (1|0|0|0)	4/50, 8%##7,^ (0|3|1|0)	1/46, 2% (0|0|1|0)	–	NTP, 2018
		Testis, seminiferous tubule degeneration Poly-3 incidence/ adjusted n, % incidence (severity profile)	NS	40/44.6, 90% (20|5|5|10)	30/40.7, 74%N* (15|5|0|10)	33/41.3, 80% (11|11|2|9)	35/42.6, 82% (9|8|4|14)	38/45.5, 84% (17|10|1|10)	33/40.6, 81% (20|3|2|8)	–	NTP, 2018
SD, 2 yr	**Pathology[4]**	**Epididymis, exfoliated germ cells** Poly-3 incidence/ adjusted n, % incidence	NS	12/39.0, 31%	15/38.3, 39%	11/37.9, 29%	13/36.1, 36%	17/39.8, 43%[6]	9/29.5, 31%	–	NTP, 2018
		Epididymis hypospermia incidence/n, %incidence (severity profile)	NS	14/49, 29% (0|0|0|14)	10/48, 21% (0|0|2|8)	15/48, 31% (0|0|2|13)	7/50, 14%N^ (0|0|0|7)	16/50, 32% (0|1|4|11)	8/46, 17% (0|0|0|8)	–	NTP, 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	250,000	
		Testis polyarteritis incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	↑*, #,^	18/49, 37% 18/38.8, 46% (7|5|5|1)	11/48, 23% 11/35.6, 31% (1|7|3|0)	15/48, 31% 15/38.0, 39% (6|4|1|4)	14/50, 28% 14/35.6, 39% (6|4|3|1)	28/49, 57%##7,^^ 28/41.8, 67%* (4|9|6|9)	16/45, 36% 16/30.7, 52% (4|7|3|2)	–	NTP, 2018

CD = continuous dose; SD = stop dose; NA = not applicable; hyphen (–) = not evaluated; d = day; yr = year; bw = bodyweight.

[1]Shading indicates statistical significance. Statistical tests varied by reference (see subsequent footnotes).

[2]↑ = positive trend, ↓ = negative trend, NS = no significant trend.

[3]Analysis performed using one-way unpaired t-test (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$); experiment performed only at high dose.

[4]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected.
Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.
Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.
Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests. All pairwise tests were one-sided and not corrected for multiple comparisons.
Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs. Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; N superscript, negative trend, or negative relative to control.

[5]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.

[6]No significant differences were found for the full analysis; however, in the sensitivity analysis that excluded all animals that overlapped with animals treated with 250,000 µg BPA/kg bw/day (see Statistical Methods), there was a significant Poly-3 test ($p = 0.046$) for the pairwise comparison of the 2,500 µg BPA/kg bw/day group to the vehicle control group (epididymis exfoliated germ cell, 16/32.4 [49%] versus 7/27.3 [26%]).

[7]SW test was nonsignificant due to the assumption of monotonicity; thus, this JT/SW data analysis result was not summarized in the CLARITY-BPA integrated report.

Chapter 9. Metabolism and Thyroid

Summarized below are findings of the CLARITY-BPA core study (NTP 2018) and the investigational study by the laboratory of Tom Zoeller (Bansal and Zoeller 2019) evaluating the potential effect of BPA on metabolism and thyroid function. Brief discussions of methods and links to the raw data from unpublished CLARITY-BPA investigational studies carried out in the laboratories of Andrew Greenberg and Nira Ben-Jonathan laboratories are also provided. All individual animal data for these studies are available online at https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA. The study details, including endpoints measured and timing of assessment, are detailed in Table 19. The statistically significant findings from the core study on metabolism and thyroid endpoints are included in Table 20.

Study Summaries

Core Study: NTP (2018)

Methods

Measurements taken in the core study that may reflect influences of BPA on metabolic processes include body weights at various ages; ovarian/parametrial, retroperitoneal and epididymal fat pad weights at 1 year; serum total protein, albumin, cholesterol, glucose, triglycerides, triiodothyronine (T3), thyroxine (T4), thyroid-stimulating hormone (TSH), insulin, and leptin at 1 year; and selected nonneoplastic lesions of the liver or other organs (e.g., liver fatty change, thyroid follicular cell hyperplasia) at 1 or 2 years.

Findings

There were few differences in body weights in BPA continuous-dose or stop-dose study animals. For continuous-dose females, mean body weights in the 250 µg BPA/kg bw/day dose group were higher by 16%–18% than those of the vehicle control group for weeks 96–104 (Table 20).

The weight of the retroperitoneal fat pad in females in the 2.5 µg BPA/kg bw/day continuous-dose group was greater than in controls in absolute measure and relative to brain weight, but not elevated in relation to body weight at 1 year. Absolute and brain-weight-adjusted and body-weight-adjusted ovarian/parametrial fat pad weights in the 25 µg BPA/kg bw/day stop-dose group were lower than vehicle controls in a sensitivity analysis that excluded all animals that overlapped (see Introduction) with the 250,000 µg BPA/kg bw/day dose group.

Liver fatty change was greater in 25 µg BPA/kg bw/day continuous-dose males than in controls at 1 year (RTE), but trended lower in BPA continuous-dosed females (JT/SW and RTE). Liver fatty change was also decreased in 2,500 µg BPA/kg bw/day stop-dose females at 2 years (JT/SW and RTE) and in continuous-dose females at 2.5 and 2,500 µg BPA/kg bw/day (Poly-3, JT/SW, and RTE) and 25,000 µg BPA/kg bw/day (JT/SW) at 2 years.

There was a trend toward decreased T4 with increasing BPA dose in the male stop-dose animals at 1 year, and T4 levels in the 25,000 µg BPA/kg bw/day group of stop-dose males were lower than in controls when excluding from analysis animals that overlapped with animals in the 250,000 µg BPA/kg bw/day group. Thyroid follicular cell hyperplasia was greater in 2.5 µg BPA/kg bw/day continuous-dose females (RTE) and in 25 µg BPA/kg bw/day continuous-dose males (JT/SW and RTE) at study termination.

Table 19. Study Details and Summary of Measured Metabolism and Thyroid Endpoints

Endpoint Category	Specific Measure	Bansal and Zoeller (2019)[1]	Ben-Jonathan (Unpublished)[2]		NTP (2018)[3]		Greenberg (Unpublished)[4]		NTP (2018)[3]	
		Age at Assessment								
		PND 15	PND 90	6 mo	1 yr				2 yr	
Body Weight[5]	Body weight	●	●	●	●	□	●	□	●	□
Organ Weight[6]	Retroperitoneal fat pad weight	–	–	–	●	□	–	–	–	–
	Subcutaneous fat pad weight	–	●	●	–	–	–	–	–	–
	Epididymal fat pad weight	–	●	●	●	□	–	–	–	–
	Ovarian/parametrial fat pad weight	–	●	●	●	□	–	–	–	–
	Thyroid weight	–	–	–	●	□	–	–	–	–
Gene Expression[7]	Brain mRNA levels	●	–	–	–	–	–	–	–	–
	Liver mRNA levels	–	–	–	–	–	●	□	–	–
	Pituitary mRNA levels	●								
	Adipose mRNA levels	–	●	●	–	–	–	–	–	–
Clinical Pathology[8]	Clinical chemistry				●	□	–	–		
	Glucose tolerance	–	–	–	–	–	●	□	–	–
	Hormone or cytokine levels	–	●	●	●	□	–	–	–	–
Pathology[9]	Cerebellum	●	–	–	–	–	–	–	–	–
	Liver	–	–	–	●	□	●	□	●	□
	Thyroid	–	–	–	●	□	–	–	●	□
	Retroperitoneal fat pad	–	–	–	●	□	–	–	●	□
	Pancreas	–	–	–	–	–	●	□	–	–
	Adipose, cellularity	–	●	●	–	–	–	–	–	–
Thyroid Function[10]	Hormone levels	●	–	–	●	□	–	–	–	–

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.
yr = year; mo = month; PND = postnatal day; bw = body weight.

[1]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose BPA treatments. Animals were sacrificed at PND 15 and examined for effects.
[2]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose BPA treatments. Animals were sacrificed at PND 90 or 6 mo and examined for effects.
[3]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr or 2 yr and examined for effects.
[4]Male and female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr and examined for effects. Animals were sacrificed also at 6 mo, but the biological samples collected were not analyzed.
[5]Endpoint list: body weight: dam, pup prewean, pup postwean ((NTP 2018)); pup body weight at necropsy (all other studies).
[6]NTP 2018 endpoint list: thyroid: absolute, relative to body weight, relative to brain weight (males and females); retroperitoneal fat pad: absolute, relative to body weight, relative to brain weight (males and females); epididymal fat pad: absolute, relative to body weight, relative to brain weight (males); ovarian/parametrial fat pad: absolute, relative to body weight, relative to brain weight (females).
Ben-Jonathan Unpublished endpoint list: absolute weight of subcutaneous fat pad, epididymal fat pad, periovarian fat pad.
[7]Bansal and Zoeller 2019 endpoint list: myelin associated glycoprotein (MAG) mRNA (corpus callosum and anterior commissure), RC3/neurogranin mRNA (hippocampus), thyrotropin releasing hormone (TRH) mRNA (hypothalamus neurons), TSHβ mRNA (pituitary).
Ben-Jonathan Unpublished endpoint list: Five categories of genes analyzed in adipose tissue: adipokines/cytokines (leptin, adiponectin, IL-6, Ccl2, and TNFα); metabolic factors (adipose triglyceride lipase, lipoprotein lipase, fatty acid synthase, glucose transporter 4, hormone sensitive lipase, and sterol regulatory binding protein); transcription factors (PPARγ and C/EBPα); receptors/aromatase (ERα, ERβ, GPR30, aromatase, insulin receptor, and prolactin receptor; housekeeping genes (β2-microglobulin and hypoxanthine phosphoribosyl transferase).
Greenberg Unpublished endpoint list: 3-hydroxy-3-methylglutaryl-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA synthase 1, acetyl-CoA carboxylase alpha, acyl-CoA thioesterase 1, adhesion G protein-coupled receptor E1 (also called F4/80), interleukin 1 beta, peptidylprolyl isomerase B (cyclophilin B), peroxisome proliferator-activated receptor alpha, peroxisome proliferator-activated receptor gamma, PPARG coactivator 1 alpha, stearoyl-CoA desaturase, sterol regulatory element binding transcription factor 1, sterol regulatory element binding transcription factor 2, tumor necrosis factor (liver).
[8]NTP 2018 endpoint list: serum levels of glucose, insulin, leptin, total protein, albumin, total bile acids, cholesterol, triglycerides.
Ben-Jonathan Unpublished endpoint list: serum levels of prolactin, leptin, adiponectin, IL-6.
Greenberg Unpublished endpoint list: serum levels of glucose, insulin.
[9]NTP 2018 endpoint list: comprehensive assessment of neoplastic and nonneoplastic lesions, including: liver: fatty change, cytoplasmic vacuolation; thyroid; c-cell hyperplasia, follicular cell hyperplasia.
Greenberg Unpublished list: steatosis score, macrosteatosis, microsteatosis (liver); by immunohistochemistry, proportion of insulin-stained beta cells, glucagon-stained alpha cells, weight of beta cells, weight of alpha cells, beta cell/pancreas weight ratio, alpha cell/pancreas weight ratio (pancreas, females only).
Bansal and Zoeller 2019 endpoint list: average thicknesses of external granule layer, mitral layer, internal granule layer (cerebellum).
[10]Bansal and Zoeller 2019 endpoint list: serum T4.
NTP 2018 endpoint list: serum levels of triiodothyronine (T3), thyroxine (T4), thyroid-stimulating hormone (TSH).

The authors stated there were no changes of biological significance in clinical chemistry parameters reflecting altered metabolism in BPA continuous- or stop-dose groups at 1 year. Thyroid c-cell hyperplasia was greater in 2.5 µg BPA/kg bw/day stop-dose females at 1 year (RTE). This lesion trended higher in continuous-dose males at terminal sacrifice (Poly-3) and was elevated in the 2,500 µg BPA/kg bw/day group of continuous-dosed males by pairwise comparisons (Poly-3, JT/SW, and RTE). Serum calcium levels were not measured in the study.

Ethinyl estradiol

In EE2-exposed females, ovarian/parametrial fat pad weight was lower in 0.5 µg EE2/kg bw/day females at 1 year (CAFE). Liver fatty change was lower than in controls with both doses of EE2 by trend analysis and pairwise comparisons of all statistical tests in females at study termination (age 2 years; Poly-3, JT-SW, and RTE). Serum TSH was greater than in controls in 0.5 µg EE2/kg bw/day females at 1 year. Thyroid follicular cell hyperplasia was greater in 0.05 µg EE2/kg bw/day females at 2 years (Poly-3 and RTE). Thyroid ultimobranchial cyst also had a positive trend in EE2-treated females at age 2 years (Poly-3).

In EE2-dosed males, decreased serum insulin compared to controls at 0.05 µg EE2/kg bw/day and increased triglycerides at 0.5 µg EE2/kg bw/day were observed at 1 year. Liver fatty change occurred with a positive trend by all statistical tests (Poly-3, JT/SW, and RTE) and was greater in males by pairwise comparisons at 0.5 µg EE2/kg bw/day (JT/SW and RTE). Thyroid c-cell hyperplasia was greater in 0.05 µg EE2/kg bw/day males at 2 years (Poly-3 and RTE).

Author conclusions

The authors did not provide specific conclusions concerning the effects of BPA exposures on metabolism or thyroid hormones, but stated that overall the findings were not "dose responsive, sometimes occurring in only one low or intermediate dose group, and did not demonstrate a clear pattern of consistent responses within or across organs within the stop- and continuous-dose arms and the interim and terminal sacrifices" (NTP 2018).

Investigational Study: Bansal and Zoeller (2019)

Methods

As a positive control for disruption of thyroid gland function, pregnant rats were assigned to either a control or 6-propyl-2-thiouracil (PTU) treatment group (eight dams per treatment) on GD 6. PTU was delivered in drinking water at a dose anticipated to reduce serum T4 by ~80%. Both control and PTU animals (dams and offspring) were dosed by gavage daily with the CMC vehicle from GD 6. One male and one female per litter were sacrificed on PND 15 for collection of trunk blood, and brain, liver, heart, and pituitary were dissected and snap frozen for shipment to the Zoeller lab. Offspring were also analyzed similarly to the control and PTU groups on PND 15 from groups of eight pregnant dams dosed by gavage with 0 (CMC control), 2.5, 25, 250, 2,500, or 25,000 µg BPA/kg bw/day. As with the PTU groups, dosing of dams began on GD 6 with pups dosed by gavage with the same doses as their dams from PND 1 to sacrifice on PND 15.

Serums were analyzed for T4 levels. Several endpoints known to be sensitive to thyroid hormone levels were measured in the brain by in situ hybridization. These included expression of RC3 neurogranin mRNA in the hippocampus, myelin associated glycoprotein (surrogate for oligodendrocyte number) mRNA in the corpus callosum and rostral anterior commissure, and

thyrotropin releasing hormone mRNA in the hypophysiotropic thyrotropin releasing hormone (TRH) neurons in the hypothalamic paraventricular nucleus. Cerebellar histogenesis was evaluated in H&E sections of PND 15 cerebellum using a reticle to measure each layer in the deepest sulcus. To assess chronic stress, corticotropin-releasing hormone mRNA was measured in the hypophysiotropic region of the hypothalamic paraventricular nucleus in gavage control PND 15 pups and compared the signal with that of sections from a matched brain region taken from naïve PND 15 Zivic Miller Sprague Dawley rats; brain tissue from Zivic Miller Sprague Dawley rat was collected in a previous study by Zoeller et al. (2005). Additional measurements on the PTU-treated pups explored serum TSH levels and pituitary TSHβ mRNA expression.

Findings

There were no significant findings reported on thyroid-related endpoints in the BPA-dosed pups of either sex; thus, the data are only presented narratively in the text (no data are included in Table 20).

In the PTU study arm, PTU reduced serum T4 to the expected degree (80% reduction) and serum TSH and pituitary TSH beta mRNA were elevated in both sexes, suggesting the pituitary-thyroid axis was functional in the NCTR-SD rat strain. Markers of thyroid function were responsive in the liver and some but not all in the heart. However, effects expected by the authors on measures of thyroid hormone action in the brain were not seen in PTU-dosed rats. For example, PTU-treated male pups showed a "relatively small" 13% reduction in RC3/neurogranin mRNA in the dentate gyrus and a "slight" but significant reduction in expression of RC3 mRNA in CA1 of the hippocampus. Other expected effects of PTU on the brain were not seen, including a lack of treatment-related effects for any endpoint measured in the brains of PTU-exposed female pups. There was no evidence of increased stress in gavage versus naïve PND 15 pups as indicated by changes in corticotropin-releasing hormone mRNA in the hypothalamic paraventricular nucleus.

Ethinyl estradiol

In similar studies with 0.05 or 0.5 μg EE2/kg bw/day, neither serum T4 levels nor any of the measured downstream endpoints were affected.

Author conclusions

The authors stated that the lack of effects of BPA with respect to thyroid hormone changes in the NCTR-SD rat were consistent with other studies in this rat strain, but that the NCTR rat was not representative of responses in other rat strains. The authors suggested that, "the finding that PTU-induced T4 suppression in NCTR-SD rats only weakly affected a subset of well-known thyroid-sensitive endpoints in male pups and no endpoints in female brain emphasizes the possibility that this particular strain is uniquely insensitive to thyroid hormone insufficiency" (Zoeller et al. 2005).

Investigational Study: Greenberg (Unpublished)

Methods

At the 1-year sacrifice, liver and pancreas were isolated and weighed. Serum and right liver lobe were flash frozen, and the left liver and pancreas were fixed before shipping to the Greenberg laboratory. Livers were further processed for H&E staining and scored for macro- and micro-steatosis. RNA was extracted from frozen liver samples, cDNA was generated, and gene

expression was measured by qPCR for peroxisome proliferator-activated receptor gamma (*Pparg),* stearoyl-CoA desaturase (*Scd1),* sterol regulatory element binding transcription factor 1 *(Srebf1),* 3-hydroxy-3-methylglutaryl-coA reductase *(Hmgcr),* 3-hydroxy-3-methylglutaryl-coA synthase 1 (*Hmgcs1),* sterol regulatory element binding transcription factor 2 *(Srebf2),* acetyl-CoA carboxylase alpha *(Acaca),* peroxisome proliferator-activated receptor alpha *(Ppara),* peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(Ppargc1a),* adhesion G protein-coupled receptor E1 (*Emr1),* interleukin 1 beta *(Il1B)* tumor necrosis factor *(Tnf),* and acyl-coA thioesterase 1*(Acot1)*.

Female pancreas sections were immunostained for insulin and glucagon to estimate beta and alpha cell volume, respectively, by scanned image analysis. Serum insulin was measured by ELISA, and glucose was measured by an enzymatic assay.

Findings

Unanalyzed data from this study are available at: https://doi.org/10.22427/NTP-DATA-018-00007-0001-000-7.

Investigational Study: Ben-Jonathan (Unpublished)

Methods

Serum was collected from animals at PND 90 and 180, frozen, and sent to the Ben-Jonathan laboratory where samples were analyzed for hormones: prolactin, leptin, adiponectin, and interleukin 6 (IL-6). Visceral adipose tissues were isolated from the epididymal fat pad in males and the ovarian/parametrial fat pads in females. Subcutaneous adipose tissue was isolated from inguinal fat pads from both sexes. Each fat pad was segmented into two parts—one fixed in formalin and the other snap frozen—and shipped to the Ben-Jonathan laboratory. For frozen tissues, five categories of genes were analyzed for expression levels using custom-designed PCR array: (1) adipokines/cytokines (leptin, adiponectin, IL-6, C-C motif chemokine ligand 2 *(Ccl2)*, and tumor necrosis factor alpha (*TNFα*); (2) metabolic factors (adipose triglyceride lipase, lipoprotein lipase, fatty acid synthase, glucose transporter 4, hormone sensitive lipase, and sterol regulatory binding protein); (3) transcription factors (peroxisome proliferator-activated receptor gamma [*PPARγ*] and CCAAT-enhancer binding protein alpha [*C/EBPα*]);
(4) receptors/aromatase (*ERα, ERβ, GPR30* [G protein-coupled estrogen receptor 1, also called G protein-coupled receptor 30], aromatase, insulin receptor, and prolactin receptor); and
(5) housekeeping genes (β2-microglobulin and hypoxanthine phosphoribosyl transferase).

For studies of adipose tissue cellularity, two parameters were assessed—adipocyte size by immunohistochemistry and macrophage infiltration by immunofluorescence.

Findings

Unanalyzed data from this study are available at: https://doi.org/10.22427/NTP-DATA-018-00005-0001-000-5.

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Table 20. Summary of Statistically Significant Metabolism and Thyroid Endpoints

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
CD, 1 yr	**Organ Weight[3]**	**Retroperitoneal fat pad weight: absolute** mean ± SE, (g) [n]	F	NS	14.1 ± 1.5 [21]	19.8 ± 2.0* [22]	16.1 ± 1.4 [21]	14.1 ± 1.1 [22]	14.1 ± 1.2 [20]	14.7 ± 1.2 [24]	NTP, 2018
		Retroperitoneal fat pad weight: relative to brain weight mean ± SE, (g/g) [n]	F	NS	6.72 ± 0.70 [21]	9.41 ± 0.97* [22]	7.64 ± 0.67 [21]	6.76 ± 0.52 [22]	6.84 ± 0.55 [20]	7.09 ± 0.56 [24]	NTP, 2018
	Clinical Pathology[4]	**Serum albumin** mean ± SE, (g/dL) [n]	M	**[5]	3.7 ± 0 [18]	3.7 ± 0 [22]	3.6 ± 0 [18]	3.6 ± 0 [24]	3.7 ± 0.1 [18]	3.7 ± 0 [21]	NTP, 2018
		Serum total bile acids mean ± SE, (µmol/L) [n]	M	↓*	32.8 ± 2.7 [18]	33.3 ± 4.1 [22]	32.5 ± 2.4 [18]	35.8 ± 3.4 [24]	42.0 ± 4.7 [18]	28.1 ± 2.7 [21]	NTP, 2018
		Serum T4 mean ± SE, (µg/dL) [n]	M	*[5]	5.0 ± 0.3 [18]	4.3 ± 0.3 [22]	5.0 ± 0.2 [18]	4.9 ± 0.2 [24]	4.7 ± 0.2 [18]	5.5 ± 0.2 [21]	NTP, 2018
	Pathology[6]	**Liver fatty change** incidence[7]/n, % incidence (severity profile)	M	NS	0/22, 0%	0/22, 0%	2/20, 10% (0|2|0|0)^	0/24, 0%	0/19, 0%	0/22, 0%	NTP, 2018
		Liver fatty change incidence/n, % incidence (severity profile)	F	↓#,^	2/23, 9% (0|1|1|0)	4/22, 18% (1|2|1|0)	2/22, 9% (1|1|0|0)	1/24, 4% (0|1|0|0)	0/20, 0%	1/24, 4% (0|0|1|0)	NTP, 2018
SD, 1 yr	**Clinical Pathology[4]**	**Serum albumin** mean ± SE, (g/dL) [n]	F	**[5]	4.1 ± 0.1 [20]	4.2 ± 0.1 [22]	4.0 ± 0.0 [20]	4.0 ± 0.1 [22]	4.0 ± 0.1 [20]	4.2 ± 0.0 [19]	NTP, 2018
		Serum total bile acids[8] mean ± SE, (µmol/L) [n]	M	↓*	36.4 ± 3.1 [20]	34.6 ± 3.2 [20]	25.0 ± 2.7 [19]*	32.6 ± 6.5 [19]	33.7 ± 4.3 [20]	35.0 ± 2.5 [22]	NTP, 2018
		Serum total protein[8] mean ± SE, (mg/dL) [n]	M	NS	7.3 ± 0.1 [20]	7.1 ± 0.1 [20]	7.0 ± 0.1 [19]*	7.2 ± 0.1 [19]	7.2 ± 0.1 [20]	7.2 ± 0.1 [22]	NTP, 2018
		Serum T4 mean ± SE, (µg/dL) [n]	M	↓*	4.9 ± 0.2 [20]	4.8 ± 0.2 [20]	4.7 ± 0.2 [19]	4.7 ± 0.2 [19]	4.7 ± 0.3 [20]	4.2 ± 0.2[9] [22]	NTP, 2018
	Pathology[6]	**Thyroid gland c-cell hyperplasia** incidence/n, % incidence (severity profile)	F	NS	10/20, 50% (10|0|0|0)	16/22, 73%^ (11|4|1|0)	11/20, 55% (8|3|0|0)	12/22, 55% (8|4|0|0)	13/20, 65% (11|2|0|0)	9/22, 41% (8|1|0|0)	NTP, 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Sex	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
				Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
CD, 2 yr	**Body Weight**	**Body weight (104 wk) mean ± SE, (g) [n]**	F		534 ± 22 [17]	619 ± 28 [18]	607 ± 18 [13]	622 ± 36* [12]	524 ± 22 [11]	597 ± 34 [9]	NTP, 2018
	Pathology[6]	**Liver fatty change** incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	F	NS	19/50, 38% 19/40.4, 47% (3|6|8|2)	9/48, 19%N#10,^ 9/35.5, 25%* (1|4|3|1)	15/46, 33% 15/32.2, 47% (2|7|6|0)	15/49, 31% 15/35.8, 42% (1|5|8|1)	8/50, 16%N##,^^ 8/31.6, 25%* (2|3|2|1)	12/46, 26%N# 12/33.5, 36% (1|5|5|1)	NTP, 2018
		Thyroid gland follicular cell hyperplasia incidence/n, % incidence (severity profile)	F	NS	1/50, 2% (0|1|0|0)	6/48, 13%^ (0|2|4|0)	4/46, 9% (0|2|1|1)	3/49, 6% (0|2|1|0)	1/50, 2% (0|0|0|1)	4/46, 9% (0|2|2|0)	NTP, 2018
		Thyroid gland c-cell hyperplasia incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	M	↑*	9/46, 20% 9/34.9, 26% (5|2|2|0)	13/40, 33% 13/32.8, 40% (5|7|1|0)	15/47, 32% 15/35.5, 42% (8|4|2|1)	15/44, 34% 15/34.1, 44% (8|6|1|0)	20/44, 45%##10,^^ 20/33.8, 59%** (10|8|2|0)	11/44, 25% 11/30.5, 36% (3|7|1|0)	NTP, 2018
		Thyroid gland follicular cell hyperplasia incidence/n, % incidence (severity profile)	M	NS	3/46, 7% (0|2|1|0)	2/40, 5% (0|1|1|0)	9/47, 19%#10,^ (0|3|5|1)	6/44, 14% (0|4|2|0)	3/44, 7% (0|1|1|1)	3/44, 7% (0|1|2|0)	NTP, 2018
SD, 2 yr	**Pathology[6]**	**Liver fatty change** incidence/n, % incidence (severity profile)	F	NS	19/49, 39% (1|5|11|2)	17/50, 34% (3|6|7|1)	17/48, 35% (3|5|7|2)	18/50, 36% (4|9|4|1)	11/50, 22%N#10,^ (2|3|4|2)	15/46, 33% (0|9|5|1)	NTP, 2018
		Thyroid gland c-cell hyperplasia incidence/n, % incidence (severity profile)	F	NS	26/48, 54% (8|15|2|1)	29/49, 59% (15|11|1|2	17/45, 38%#10,^ (11|5|0|1)	23/48, 48%#10 (15|7|1|0)	28/50, 56% (10|12|4|2)	24/46, 52% (11|11|2|0)	NTP, 2018

CD = continuous dose; SD = stop dose; bw = body weight.
[1]Shading indicates statistical significance. Statistical tests varied by reference (see subsequent footnotes).
[2]↑ = positive trend, ↓ = negative trend, NS = no significant trend.
[3]Organ weights were collected at interim sacrifice (1 yr) only. ANOVA (followed by Dunnett's test) was used to determine the effect of treatment on absolute organ weight. Separate ANOCOVA (followed by Dunnett's test) was used to determine the effect of treatment on organ weight adjusted for body weight or brain weight (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).
[4]Orthogonal contrasts were used to test for trend (*, statistically significant at $p \leq 0.05$; **, statistically significant at $p \leq 0.01$).
[5]Trend direction is unclear.

[6]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected.
Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.
Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.
Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests. All pairwise tests were one-sided and not corrected for multiple comparisons.
Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs.
Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; N superscript, negative trend, or negative relative to control.
[7]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.
[8]A nonparametric ANOVA based on mid-ranks was used to evaluate the effect of treatment. Dunnett's adjustment was used for pairwise multiple comparisons relative to the control.
[9]T4 at 25,000 µg BPA/kg bw/day significantly different from vehicle control ($p = 0.015$) in the sensitivity analysis that excluded all animals that overlapped with animals treated with 250,000 µg BPA/kg bw/day.
[10]SW test was nonsignificant due to the assumption of monotonicity; thus, this JT/SW data analysis result was not summarized in the CLARITY-BPA integrated report.

Chapter 10. Uterus

Summarized below are findings for the CLARITY-BPA core study (NTP 2018) and the investigational study by the laboratory of Shuk-Mei Ho (Leung et al. 2020) with respect to the potential effects of BPA on the uterus. All individual animal data for both of these studies are available online at https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA. The study details, including endpoints measured and timing of assessment, are detailed in Table 21. The statistically significant findings from the core study on the uterus are included in Table 22.

Table 21. Study Details and Summary of Measured Uterus Endpoints

Endpoint Category	Specific Measure	Leung et al. (2020)[1]	Ho (Unpublished)[1]	Leung et al. (2020)[1]	NTP (2018)[2]			
		Age at Assessment						
		PND 21	PND 90	6 mo	6 mo	1 yr	1 yr	2 yr
Gene Expression[3]	Uterus RNA sequencing	–	–	–	–	●	– –	– –
Organ Weight[4]	Uterus weight	●	●	●	●	●	● □	– –
Pathology[5]	Uterus	●	●	●	●	●	● □	● □

● = continuous dose; □ = stop dose; hyphen (–) = not evaluated.
PND = postnatal day; mo = month; yr = year; bw = body weight.
[1]Female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose BPA treatment. Animals were sacrificed at PND 21, PND 90, 6 mo, and 1 yr and examined for effects.
[2]Female Sprague Dawley rats were exposed via gavage at 0, 2.5, 25, 250, 2,500, or 25,000 µg/kg bw/day using continuous-dose or stop-dose BPA treatments. Animals were sacrificed at 1 yr or 2 yr and examined for effects.
[3]Leung et al. 2020 endpoint list: whole-genome RNA-seq counts.
[4]Leung et al. 2020 endpoint list: uterus weight: absolute.
NTP 2018 endpoint list: uterus weight: absolute, relative to body weight, relative to brain weight.
[5]Ho unpublished endpoint list: histology, percentage of cells positive for proliferating cell nuclear antigen, percentage of apoptotic cells. Data are available in CEBS at https://doi.org/10.22427/NTP-DATA-018-00008-0001-000-8.
Leung et al. 2020 endpoint list: histology, percentage of cells positive for proliferating cell nuclear antigen, percentage of apoptotic cells in PND 21, PND 90, and 1-year-old samples.
NTP 2018 endpoint list: comprehensive assessment of neoplastic and nonneoplastic lesions including stromal polyps, hyperplasia in endometrium, lumen dilatation, and squamous metaplasia.

Study Summaries

Core Study: NTP (2018)

Methods

The endpoints evaluated in the core study related to the uterus were blotted uterine weights evaluated at 1 year and histopathology evaluated at 1 year (interim sacrifice) and 2 years (terminal sacrifice).

Findings

In the continuous-dose arm at 1 year, the incidence of stromal polyps occurred with positive trends (CAFE) (Table 22). At 2 years, the incidence of stromal polyps in the stop-dose females occurred with a negative trend among the treatment groups, and incidence in the 25,000 µg BPA/kg bw/day dose group was lower compared to controls (Poly-3).

In the continuous-dose arm at 1 year, the incidence of endometrial hyperplasia was greater in the 2.5 and 250 μg BPA/kg bw/day dose groups compared to control (RTE) (Table 22). Apoptosis in the luminal epithelial cells in the 25,000 μg BPA/kg bw/day dose group was greater compared to controls (CAFE, JT/SW, and RTE). Apoptosis and squamous metaplasia also occurred with positive trends (CAFE, JT/SW, and RTE). In addition, the incidence of squamous metaplasia was greater at the 25,000 μg BPA/kg bw/day dose level compared to control in a sensitivity analysis that excluded a subset of animals that were housed in the same room as a special 250,000 μg BPA/kg bw/day dose group (CAFE).

In the stop-dose arm at 1 year, there was an increase in dilatation of the lumen in the 250 μg BPA/kg bw/day dose group compared to control (RTE). At the highest dose group, 25,000 μg BPA/kg bw/day, incidences of squamous metaplasia and cystic endometrial hyperplasia were also increased by pairwise comparisons (JT/SW and RTE).

In the continuous-dose arm at 2 years, cystic endometrial hyperplasia was reduced compared to control in the 2.5 μg BPA/kg bw/day dose group (Poly-3 and RTE). Dilatation of the lumen also occurred with positive trends (Poly-3, JT/SW, and RTE) and was increased in the 25,000 μg BPA/kg bw/day dose group in a pairwise comparison sensitivity analysis that excluded all animals that overlapped with animals in a special 250,000 μg BPA/kg bw/day dose group (Poly-3).

In the stop-dose group at 2 years, the incidence of squamous metaplasia was reduced in the 2.5 μg BPA/kg bw/day dose group compared to control (RTE). Atrophy was also reduced in the 25 μg BPA/kg bw/day dose group compared to control (RTE). Cystic endometrial hyperplasia occurred with positive trends (JT/SW and RTE) and was increased by pairwise comparisons in the 2,500 μg BPA/kg bw/day (JT/SW and RTE) and 25,000 μg BPA/kg bw/day (JT/SW) dose groups. Hyperplasia in the endometrium and dilatation of the lumen both occurred with negative trends (Poly-3 and Poly-3, JT/SW, and RTE, respectively).

Ethinyl estradiol

Following continuous exposure to 0.5 μg EE2 kg bw/day, the incidences of uterine apoptosis, cystic endometrial hyperplasia, and squamous metaplasia were increased by trend tests and pairwise comparisons compared to control at 1 year (CAFE, JT/SW, and RTE). At 2 years, atrophy in the high dose was greater compared to control and occurred with positive trends (Poly-3, JT/SW, RTE), and the incidence of endometrial hyperplasia was greater in the 0.05 μg EE2/kg bw/day dose group compared to control (Poly-3 and RTE). Endometrial cysts (Poly-3) and squamous metaplasia (Poly-3 and JT/SW) occurred with positive trends at 2 years.

Author conclusions

The study authors concluded that the relatively small increased incidences of stromal polyps leading to the significant trend in the animals at 1 year together with the lack of any effect in the animals at 2 years indicate that this effect is not likely to be a consequence of BPA treatment. However, the remaining effects reported, particularly from the animals at 1 year, suggest that the 25,000 μg BPA/kg bw/day dose may exert weak estrogen-like effects on the uterus.

Investigational Study: Leung et al. (2020)

Methods

Uterine weight and histopathology were evaluated in females from the continuous-dose arm of the study at PND 21, PND 90, 6 months (Ho, unpublished; available in CEBS) and 1 year. Estrous cycle status was determined by vaginal cytology at PND 90, 6 months, and 1 year, and uterine tissues from PND 90, 6 months, and 1-year-old animals were collected when predicted to be in estrus, with the actual stage determined at sacrifice. Uteri were divided longitudinally. The left half was flash frozen for storage until processing for whole-genome expression analysis by RNA sequencing, and the right half was preserved in formalin for histology and immunochemistry. Frozen and fixed uterine samples were shipped to the Ho laboratory. Histological evaluation of the right half of the uterus surveyed pathological findings (e.g., hyperplasia, squamous metaplasia), and immunohistochemical analyses measured the percentage of proliferating cells and percentage of apoptotic cells.

RNA was sequenced at the Genomics, Epigenomics and Sequencing Core at the University of Cincinnati. Data were analyzed using hierarchical clustering on pairwise comparisons, content-specific Bayesian clustering analysis, and multidimensional scaling analysis to identify similar gene signatures across BPA groups that were distinct from the control group. Common genes across BPA groups were further analyzed to predict upstream regulators and specific directions of effect by BPA. The genes were then compared to RNA data from The Cancer Genome Atlas uterine corpus endometrial carcinoma cohort to determine if the common genes found in the BPA groups are associated with poor overall survival of humans with endometrial cancers.

Two predictive models (generalized logistic regression model [GLRM] and Fisher's linear discriminant analysis [LDA]) were employed to combine estrous status, pathology (yes/no), and immunohistochemical staining for proliferating cell nuclear antigen and apoptosis data to categorize the combined response of each BPA dose group as being more similar to the low or high EE2 groups or the controls.

Findings

The results from individual endpoint evaluations described in this section did not reach statistical significance and therefore are not included in the summary table of statistically significant findings. A narrative summary of the findings is presented below followed by the results of an integrated analysis.

Continuous exposure to BPA did not affect uterus weight or histology at PND 21, PND 90, or 1 year, although there was a low incidence of squamous metaplasia in the 25 and 25,000 µg BPA/kg bw/day exposure groups compared with controls at 1 year that did not reach statistical significance. At PND 21, rats exposed to 2.5 or 2,500 µg BPA/kg bw/day exhibited more apoptotic cells in the cycling endometrium than in control females; however, those differences were not observed at PND 90 or 1 year. In addition, there was a non-statistically significant decrease in proliferation of uterine epithelial cells in the 250 µg BPA/kg bw/day dose group at 1 year and minor, non-statistically significant effects on estrous cyclicity in the 250, 2,500, and 25,000 µg BPA/kg bw/day dose groups compared with controls in 1-year-old animals.

In uteri from BPA-exposed and control 1-year-old rats in estrus, there were more than 400 genes differentially expressed in each of the BPA-exposed groups relative to controls. In a content-

specific Bayesian clustering analysis of the top 1,000 significantly differentially expressed (false discovery rates [FDR] < 0.1) genes, those from uteri of rats exposed to 25 or 250 µg BPA/kg bw/day formed tighter clusters with one another and shared similar nodes in the dendrogram (see Figure 8, panel A). Analysis of the level of similarity between individual samples, based on the expression level of all genes (using a multidimensional scaling analysis), showed that differentially expressed genes in the 25 and 250 µg BPA/kg bw/day dose groups were closely related to each other but were distinct from the control and EE2 groups (see Figure 8, panel B). Knowledge-based gene ontology analysis of the transcriptomic data derived from the 25 and 250 µg BPA/kg bw/day dose groups showed that 710 genes were common to both groups but did not overlap with the other groups (see Figure 9). Further, 262 of 710 genes showed a greater fold difference in the 25 µg BPA/kg bw/day dose group compared with the 250 µg BPA/kg bw/day dose group. Finally, Ingenuity Pathways Analysis found that 115 of the 710 BPA-responsive genes were predicted to be associated with estrogen, and "beta-estradiol" was identified as a key upstream regulator.

The data from 6-month-old females are not published in a peer-reviewed journal. The unanalyzed data are available in CEBS database at https://doi.org/10.22427/NTP-DATA-018-00008-0001-000-8.

Ethinyl estradiol

Female rats exposed to high-dose EE2 (0.5 µg/kg bw/day) had significantly higher absolute and normalized-to-body weight uterus weights compared with controls at PND 21. The incidence of squamous metaplasia was higher in the 0.5 µg EE2/kg bw/day dose group than in controls at PND 90. There was also a difference in the distribution of estrous status in the high-dose EE2 group compared with controls at 1 year. Slight increases in uterine epithelial cell proliferation and cycling endometrial apoptosis were observed at PND 21, and a low incidence of squamous metaplasia occurred at 1 year in the high-dose group, although none of these effects reached statistical significance. Uterine genes from the 0.05 and 0.5 µg EE2/kg bw/day dose groups also shared more similar gene expression patterns with the 2.5, 2,500, and 25,000 µg BPA/kg bw/day dose groups and control group when the transcriptomes were analyzed using a content-specific Bayesian clustering of the top 1,000 significant genes.

With the exception of transcriptomics data, individual evaluation of BPA on uterine endpoints did not show statistically significant effects. However, GLRM and Fisher's LDA were performed, combining all measured parameters collected at 1 year as predictors, in order to identify which dose(s) of BPA acted similarly to EE2. Samples collected from 25 and 250 µg BPA/kg bw/day dose groups showed higher propensity scores (GLRM) and smaller distances (LDA) from the high-dose EE2 group, indicative of greater similarity to the high dose of EE2. In contrast, the other BPA groups (2.5, 2,500, and 25,000 µg BPA/kg bw/day) and the low-dose EE2 group (0.05 µg/kg bw/day) were more likely to be similar to the control group.

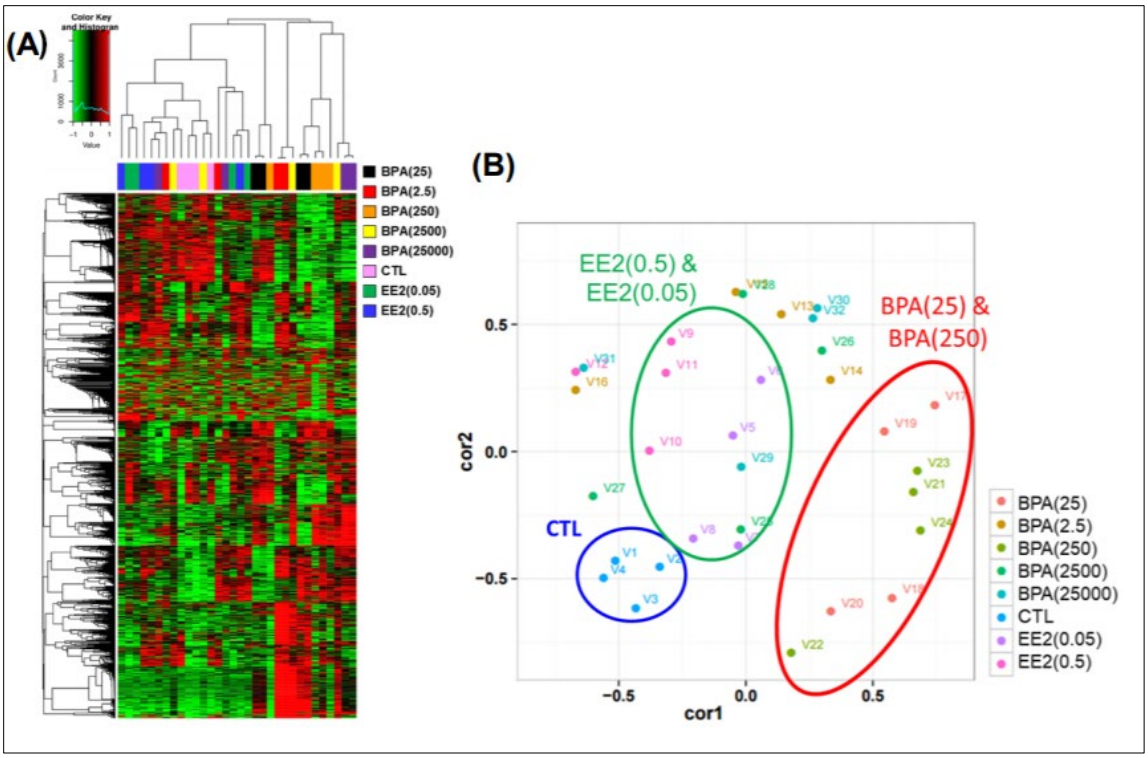

Figure 8. Transcriptomic RNA-seq Analysis of Uterus Samples at the Estrus Stage After 1-Year BPA Exposure

Transcriptomic analysis was conducted on uterus samples at estrus (n = 4) using RNA-seq. (A) The top 1,000 genes with significant differences (FDR < 0.1) compared with controls were analyzed with a Bayesian infinite mixture model, and the samples were analyzed with average-linkage hierarchical clustering and presented as a heatmap; rows indicate individual genes and columns indicate individual samples. Treatment groups in columns are color-coded. (B) All genes were subjected to multidimensional scaling analyses to visualize the similarity between individual samples. Samples are color-coded by groups; some groups are circled to show the proximity of each sample in a particular group. [Reprinted with permission from Leung et al. (2020).]

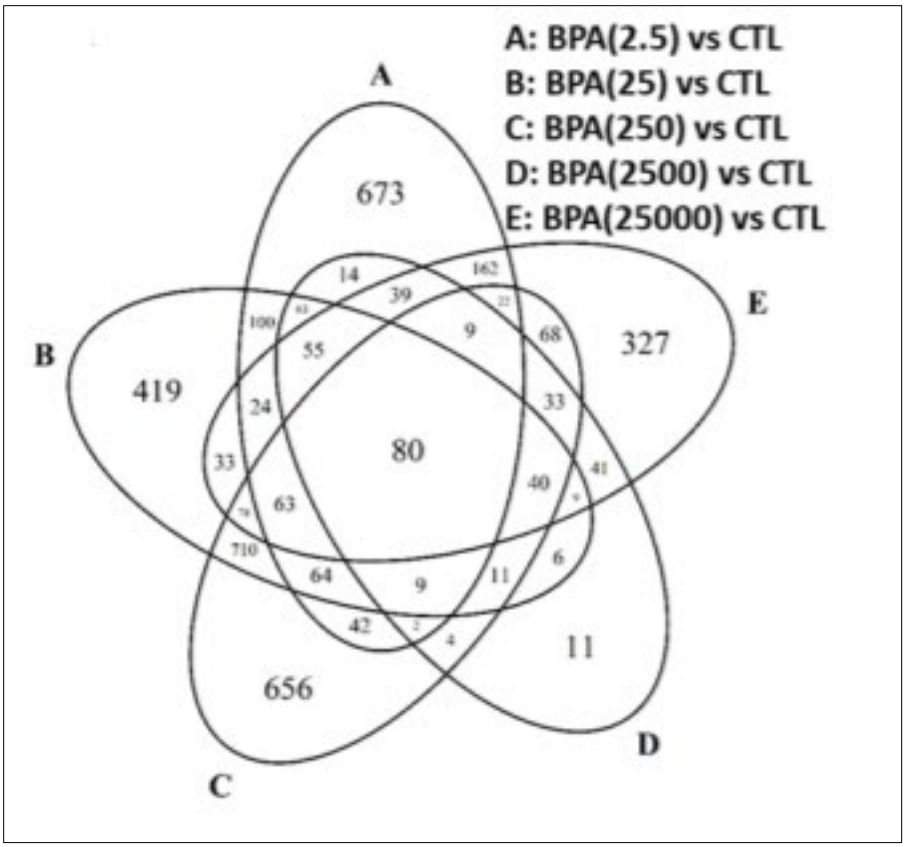

Figure 9. Venn Diagram of Differentially Expressed Genes from Uterine Samples Collected at Estrus

Transcriptomic analysis was conducted on uterus samples at estrus using RNA-seq. Significantly differentially expressed genes (FDR < 0.1 versus controls) are shown in the Venn diagram. [Reprinted with permission from Leung et al. (2020).]

Author conclusions

Although no statistically significant effects were observed when uterine endpoints were analyzed individually, the authors concluded that "life-long exposure to low doses of BPA (25 or 250 µg BPA/kg [bw]/day) but not higher doses, altered the estrous cycle, associated transcriptomes, and uterine pathology" when the combined effects of all testing outcomes were analyzed in a semi-blind fashion. Further, a subset of BPA-responsive genes from the 25 and 250 µg BPA/kg bw/day dose groups was linked to estrogen and "exhibited a non-monotonic dose-response pattern." In additional data analyses not summarized here, a subset of 57 BPA-responsive genes was associated with human cervical cancer in a disease prediction analysis (Leung et al. 2020).

Table 22. Summary of Statistically Significant Uterus Endpoints

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
CD, 1 yr	Pathology[3]	**Stromal polyps** incidence[4]/n %	↑*	1/23, 4%	0/22, 0%	1/21, 5%	0/24, 0%	3/20, 15%	3/24, 13%	NTP, 2018
		Apoptosis incidence/n, % incidence (severity profile)	↑**,##,^^	2/23, 9% (0|0|0|2)	1/22, 5% (0|0|0|1)	4/21, 19% (0|1|1|2)	5/24, 21% (0|1|0|4)	5/20, 25% (0|0|3|2)	9/24, 38%*,#,^^ (0|1|5|3)	NTP, 2018
		Hyperplasia in endometrium incidence/n, % incidence (severity profile)	NS	2/23, 9% (0|2|0|0)	7/22, 32%^ (3|3|1|0)	5/21, 24% (0|4|1|0)	7/24, 29%^ (2|4|1|0)	5/20, 25% (1|2|2|0)	2/24, 8% (0|2|0|0)	NTP, 2018
		Squamous metaplasia incidence/n, % incidence (severity profile)	↑*,#,^	1/23, 4% (0|1|0|0)	1/22, 5% (0|1|0|0)	4/21, 19% (3|1|0|0)	3/24, 13% (3|0|0|0)	3/20, 15% (2|0|1|0)	5/24, 21%[5] (5|0|0|0)	NTP, 2018
SD, 1 yr		**Cystic hyperplasia in endometrium** incidence/n, % incidence (severity profile)	NS	2/20, 10% (0|1|0|1)	4/22, 18% (0|2|2|0)	2/20, 10% (0|2|0|0)	2/22, 9% (0|2|0|0)	1/20, 5% (0|0|0|1)	7/22, 32%#,^ (2|4|0|1)	NTP, 2018
		Squamous metaplasia incidence/n, % incidence (severity profile)	NS	0/20, 0% (–)	2/22, 9% (2|0|0|0)	1/20, 5% (0|1|0|0)	1/22, 5% (1|0|0|0)	0/20, 0% (–)	4/22, 18%##,^^ (1|3|0|0)	NTP, 2018
		Lumen dilatation incidence/n, % incidence (severity profile)	NS	1/20, 5% (0|0|0|1)	0/22, 0% (–)	1/20, 5% (0|0|0|1)	4/22, 18%^ (0|0|1|3)	1/20, 5% (0|0|1|0)	0/22, 0% (–)	NTP, 2018
CD, 2 yr		**Lumen dilatation** incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	↑*,#,^	2/50, 4% 2/35.6, 6% (0|0|0|2)	2/48, 4% 2/33.5, 6.0% (0|0|0|2)	3/45, 7% 3/28.5, 11% (0|0|0|3)	4/49, 8% 4/34.5, 12% (0|0|1|3)	5/48, 10% 5/30.1, 17% (0|0|0|5)	6/46, 13.0% 6/33.0, 18%[6] (0|0|1|5)	NTP, 2018
		Cystic hyperplasia in endometrium incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	NS	30/50, 60% 30/41.9, 72% (4|18|5|3)	20/48, 42%N^ 20/40.1, 50%N* (2|13|5|0)	26/45, 58% 26/36.9, 70% (0|16|7|3)	23/49, 47% 23/40.6, 57% (4|12|6|1)	22/48, 46% 22/35.8, 61% (4|8|8|2)	26/46, 57% 26/39.2, 66% (1|14|10|1)	NTP, 2018
SD, 2 yr		**Stromal polyps** Poly-3 incidence/ adjusted n, % incidence	↓*	7/33.0, 21%	4/32.3, 12%	5/32.5, 15%	6/35.9, 17%	4/35.8, 11%	1/31.0, 3%N*	NTP, 2018
		Atrophy incidence/n, % incidence (severity profile)	NS	10/49, 20% (0|0|7|3)	6/49, 12% (0|0|5|1)	4/48, 8%N^ (0|0|4|0)	7/49, 14% (0|0|6|1)	7/49, 14% (0|0|6|1)	9/46, 20% (0|0|8|1)	NTP, 2018

Dose Type, Assessment Age	Endpoint Category	Specific Endpoint	Results by BPA Dose Level (µg/kg bw/day)[1]							Reference
			Trend[2]	Vehicle	2.5	25	250	2,500	25,000	
		Lumen dilatation incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	↓*,#,^	3/49, 6% 3/32.5, 9% (0|0|1|2)	6/49, 12% 6/33.0, 18% (0|0|0|6)	2/48, 4% 2/31.3, 6% (0|0|0|2)	4/49, 8% 4/33.8, 12% (0|0|0|4)	2/49, 4% 2/35.2, 6% (0|0|1|1)	0/46, 0% 0/30.9, 0% (–)	NTP, 2018
		Cystic hyperplasia in endometrium incidence/n, % incidence (severity profile)	↑#,^	18/49, 37% (3|8|5|2)	23/49, 47% (1|18|4|0)	22/48, 46% (1|15|5|1)	25/49, 51% (3|15|6|1)	28/49, 57%#,^ (0|18|6|4)	24/46, 52%# (4|11|6|3)	NTP, 2018
		Hyperplasia in endometrium incidence/n, % incidence Poly-3 incidence/ adjusted n, % incidence (severity profile)	↓*	18/49, 37% 18/38.8, 46% (6|12|0|0)	14/49, 29% 14/37.4, 38% (8|6|0|0)	17/48, 35% 17/37.0, 46% (7|9|0|1)	14/49, 29% 14/39.2, 36% (6|7|1|0)	12/49, 24% 12/38.5, 31% (2|10|0|0)	10/46, 22% 10/33.6, 30% (1|8|1|0)	NTP, 2018
		Squamous metaplasia incidence/n, % incidence (severity profile)	NS	5/49, 10% (3|2|0|0)	1/49, 2%N#7,^ (1|0|0|0)	2/48, 4% (1|1|0|0)	2/49, 4% (0|2|0|0)	4/49, 8% (3|1|0|0)	3/46, 7% (3|0|0|0)	NTP, 2018

CD = continuous dose; SD = stop dose; bw = body weight.

[1]Shading indicates statistical significance. Statistical tests varied by reference (see subsequent footnotes).

[2]↑ = positive trend, ↓ = negative trend, NS = no significant trend.

[3]Statistical analyses were conducted for any lesion that was diagnosed in two animals in any dose group in the interim sacrifice groups or four animals in the control and BPA groups in the terminal sacrifice groups. Incidence represents number of animals with lesions/number of animals examined microscopically and percent animals affected. Severity profile represents the number of animals diagnosed with minimal | mild | moderate | marked lesions.

Lesions in interim sacrifice animals were analyzed by the CAFE (Cochran-Armitage trend test and Fisher's exact) tests (conducted on simple incidence data) to compare the trend across dose groups and the pairwise comparisons of incidence in each dose group to the vehicle control and by the Jonckheere-Terpstra (JT) trend test/Shirley-Williams (SW) pairwise comparison test to incorporate severity scores. Because the JT/SW test enforces an assumption of a monotonic response, a relative treatment effect (RTE) analysis that also incorporates severity scores, but does not enforce monotonicity, was also conducted. All pairwise tests were one-sided and not corrected for multiple comparisons.

Lesions in terminal sacrifice animals were analyzed by the Poly-3 test to adjust for intercurrent mortality and by the JT/SW and RTE tests. All pairwise tests were one-sided and not corrected for multiple comparisons.

Significant findings for the CAFE or Poly-3 tests for interim and terminal sacrifice animals, respectively, are indicated by asterisk (*), JT/SW test by pound (#), and RTE test by caret (^) signs.

[4]For the interim sacrifice and terminal sacrifice postweaning analyses, there were no littermates among the males or females in any dose group within each dosing arm and sacrifice time, so intra-litter correlation was not considered. Incidence is based on individuals.

Significance markers: *, #, ^, statistically significant at $p \leq 0.05$; **, ##, ^^, statistically significant at $p \leq 0.01$; N superscript, negative trend, or negative relative to control.

[5]In the sensitivity analysis that excluded all animals that overlapped with animals treated with 250,000 µg BPA/kg bw/day, there was a significant ($p = 0.048$) difference for the CAFE test for the pairwise comparison of the 25,000 µg BPA/kg bw/day group to the vehicle control (squamous metaplasia, 5/20 [25%] versus 0/15 [0%]).

[6]No significant differences were found for pairwise comparisons in the full analysis; however, in the sensitivity analysis that excluded all animals that overlapped with animals treated with 250,000 µg BPA/kg bw/day (see Statistical Methods), there was a significant Poly-3 test ($p = 0.035$) for the pairwise comparison of the 25,000 µg BPA/kg bw/day group to the vehicle control group (lumen dilatation, 5/26.8 [19%] versus 0/24 [0%]).

[7]SW test was nonsignificant due to the assumption of monotonicity; thus, this JT/SW data analysis result was not summarized in the CLARITY-BPA integrated report.

Concluding Remarks

In summary, all data from the core study and the majority of data from the grantee studies, including unpublished studies, are available in the NTP CEBS database (https://cebs.niehs.nih.gov/cebs/program/CLARITY-BPA). In addition, readers can reference the peer-reviewed publications for more details on the methods, findings, and interpretation of the respective CLARITY-BPA authors. While the current report does not include an overall integration of the findings or integration of the conclusions of these CLARITY-BPA program publications, the data are presented for other researchers to consider this effort.

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Appendix A. CLARITY-BPA Compendium Report Gene Glossary

Abbreviation	Gene Name
Acaca	Acetyl-CoA carboxylase alpha
Acot1	Acyl-CoA thioesterase 1
Ar	Androgen receptor
Avp	Arginine vasopressin
Avpr1a	Arginine vasopressin receptor 1A
B2m	Beta-2 microglobulin
Bdnf	Brain-derived neurotrophic factor
Bmp4	Bone morphogenetic protein 4
Camk4	Calcium/calmodulin-dependent protein kinase 4
Ccl2	Chemokine (C-C motif) ligand 2
Ccl6	Chemokine (C-C motif) ligand 6
Cebpa	CCAAT/enhancer binding protein alpha
Chga	Chromogranin A
CK5	Cytokeratin 5
CK8	Cytokeratin 8
Cyp11a1	Cytochrome P450 family 11 subfamily A polypeptide 1
Cyp19a1	Cytochrome P450 family 19 subfamily A member 1
Dkk2	Dickkopf WNT signaling pathway inhibitor 2
Dnmt1	DNA methyltransferase 1
Dnmt3a	DNA methyltransferase 3 alpha
Dnmt3b	DNA methyltransferase 3 beta
Emr1	Adhesion G protein-coupled receptor E1
Esr1	Estrogen receptor 1 (ER-alpha)
Esr2	Estrogen receptor 2 (ER-beta)
Erra/Esrra	Estrogen receptor-related alpha

Errb	Estrogen receptor-related beta
Esr1	Estrogen receptor 1
Esr2	Estrogen receptor 2
Gadd45b	Growth arrest and DNA damage inducible beta
Gpr30	G protein coupled receptor 30
Grm5	Glutamate metabotropic receptor 5
Hmgcr	3-hydroxy-3-methylglutaryl-CoA reductase
Hmgcs1	3-hydroxy-3-methylglutaryl-CoA synthase 1
Hoxb13	Homeobox protein B13
Hprt	Hypoxanthine phosphoribosyl transferase
Igf1	Insulin-like growth factor 1
Il1b	Interleukin 1 beta
Il6	Interleukin 6
Lepr	Leptin receptor
Mag	Myelin associated glycoprotein
Nr5a1	Nuclear receptor subfamily 5 group A member 1 (see also Sf-1)
Otr/Oxtr	Oxytocin receptor
Oxt	Oxytocin
Ppara	Peroxisome proliferator-activated receptor alpha
Pparg	Peroxisome proliferative activated receptor gamma
Ppargc1a	Peroxisome proliferative activated receptor gamma coactivator 1 alpha
Ppib	Peptidylprolyl isomerase B
Ptgds	Prostaglandin D2 synthase
Scd1	Stearoyl-Coenzyme A desaturase
Sf1	Steroidogenic factor 1
Sfrp4	Secreted frizzled-related protein 4
Slc1a2	Solute carrier family 1 member 2
Slc2a4	Glucose transporter 4 (Solute carrier family 2 (facilitated glucose transporter), member 4)

Slc32a1	Solute carrier family 32 member 1
Sox2	Sex-determining region Y box 2
Sox9	Sex-determining region Y box 9
Srebf1	Sterol regulatory element binding transcription factor 1
Srebf2	Sterol regulatory element binding transcription factor 2
Tbx3	T-box 3
Thbs2	Thrombospondin 2
Tnf	Tumor necrosis factor
Tnfa	Tumor necrosis factor alpha
Trh	Thyrotropin releasing hormone
Trop2	Tumor-associated calcium signal transducer 2
Tshβ	Thyroid hormone stimulating hormone beta

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