Thus, our analyses using our ES cell differentiation system indicate that CBZ and VPA exert different embryotoxic effects because of the differential sensitivities of endodermal and mesodermal developmental stages to these drugs. Since VPA inhibits the expression of the examined markers through all stages of endodermal and mesodermal development, VPA would be expected to display a greater embryotoxic effect than CBZ, which inhibits the expression of markers only at later stages of endodermal and mesodermal development.

In culture medium containing 20% FCS, ES cells mainly differentiated into mesodermal and endodermal lineages, but not into ectodermal lineages such as neural cells. However, in previous report, VPA induced neural differentiation in a lineage-specific manner [14]. Therefore, we examined the expression levels of various neural markers: Nestin, which is a marker of early differentiation; and Synaptophysin (Syn) and Neurofilament H (NFH), which are later stage neuron-specific markers. The expression of all three markers increased in a dose-dependent manner in the presence of CBZ (Fig. 2A-1/2). The glial markers GFAP, an astrocyte-specific marker, and Oligo2 and DM20, oligodendrocyte-specific markers, were also elevated in a concentration-dependent manner (Fig. 2A-3). These results suggest that CBZ induces ES cells to differentiate into neurons and glial cells. This contrasts with our observation in the previous study that VPA induces ES cells to differentiate into neurons but not glial cells [14]. In order to compare the expression profiles of neuronal and glial markers between CBZ and VPA, we performed RT-PCR with samples on days 5 and 7. RT-PCR analysis showed that the Nestin expression levels induced by CBZ were higher than those induced by VPA (Fig. 2B). Syn and NFH showed higher levels of expression after CBZ than VPA at day 5, but no expression of either marker could be detected at day 7 in the CBZ cultures. In contrast, the expression of Syn and NFH induced by VPA was increased in the day 7 culture. The DM20 expression levels in CBZ cultures were higher on days 5 and 7 than in the VPA cultures. The expression levels of other typical glial markers, such as GFAP (a representative astrocyte-specific marker), and Olig2 (another oligodendrocyte-specific marker), were very low (data not shown). Our immunocytochemical study with an antibody against β-III tubulin (a neuronal marker) revealed that many positive cells were detected in samples on day 10 of cultures with either 0.05 mM CBZ (Fig. 2C, left) or 0.19 mM VPA (Fig. 2C, right). In the CBZ-administered group, the positive cells had an almost spherical shape and few had nerve processes. These cells had the appearance of immature neurons. In contrast, in the VPA-administered group, many of the positive cells had long nerve processes and had the appearance of mature neurons. These results suggest that: (1) CBZ induces neural lineage differentiation in ES cells but that the potential for neuronal differentiation is lower compared with VPA; (2) CBZ induces differentiation of both neuronal and glial lineages, whereas VPA induces neuronal but not glial cells [14]. The embryotoxic effects of VPA in humans are mainly due to a failure of the neural tube to close (neural tube defects, NTDs), leading to conditions

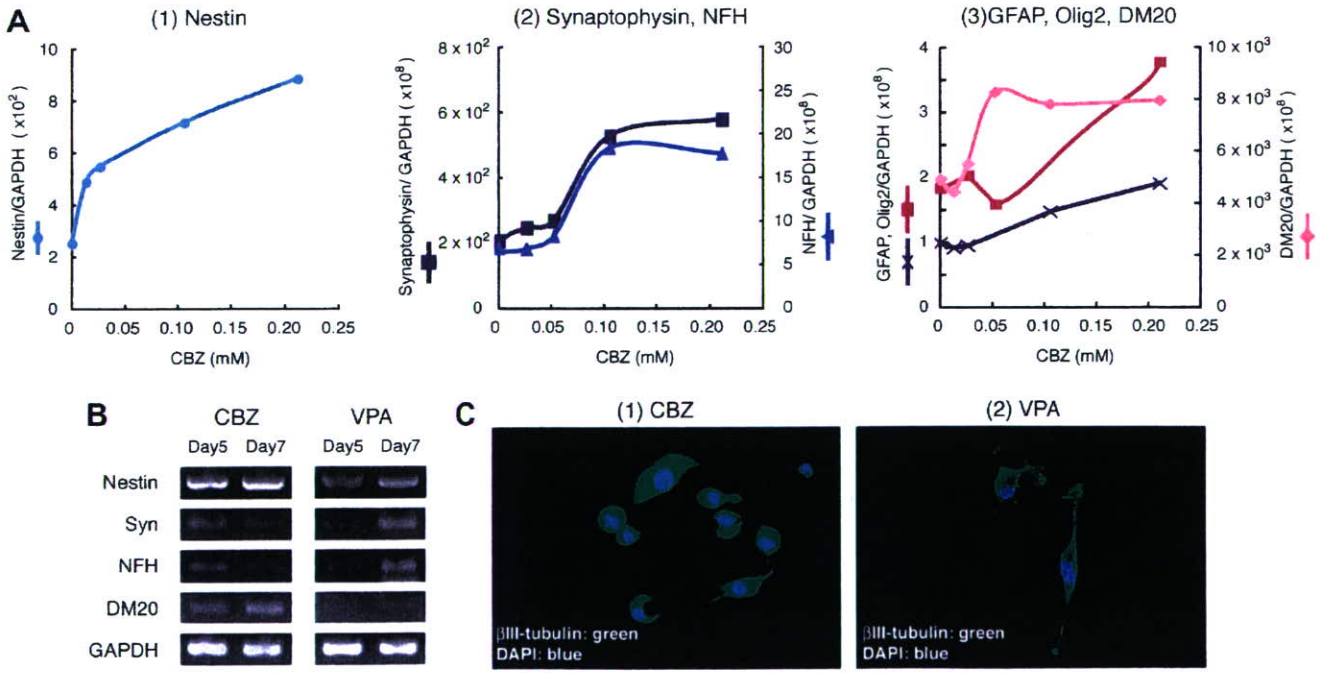

Fig. 2. CBZ embryotoxicity in the differentiating ectodermal lineage. (A) Gene expression levels of the ectodermal markers, Nestin (1), Synaptophysin, NFH (2), and GFAP, Oligo2, DM20 (3), were quantified by real-time RT-PCR. (B) The expression levels of ectodermal markers were quantified by RT-PCR in cultures treated with CBZ (0.11 mM) or VPA (1.50 mM). (C) Neuronal cells derived from ES cells cultured in the presence of CBZ (0.05 mM) or VPA (0.19 mM) were immunostained with an anti-βIII tubulin antibody.

such as spina-bifida-aperta, anencephaly, and exencephaly [18]. CBZ also induces several NTDs but the frequency and severity of the abnormalities induced by CBZ are lower than by VPA [13]. Thus, the differences found between these two drugs in our *in vitro* assay matches the differences found after *in vivo* use of the drugs.

Cytotoxic effects of CBZ

The cell viability assay (MTT assay) was used to study the cytotoxic effect of CBZ on ES cells (representing embryonic tissues) and NIH-3T3 fibroblasts (representing adult tissues). In both cell lines, CBZ inhibited the survival of cells in a dose-dependent manner (Fig. 3). There was no significant difference between the cytotoxic sensitivities of ES cells and NIH-3T3 fibroblasts to CBZ. The IC_{50} values, the inhibitory concentration giving 50% cell viability, were calculated as 0.24 and 0.31 mM for NIH-3T3 fibroblasts and ES cells, respectively. The therapeutic range of CBZ is 0.02–0.05 mM in serum. Thus, the IC_{50} values of NIH-3T3 fibroblasts and of ES cells were approximately 5–15 times larger than the therapeutic concentration. Neither cell line appears to show any significant response to CBZ within the therapeutic range. The cytotoxicity of CBZ for ES cells was much lower than obtained for VPA in our previous study [14]. VPA has an IC_{50} value of 3.25 mM for NIH-3T3 fibroblasts, which is approximately 5–11 times the therapeutic range of VPA, 0.30–0.70 mM. The IC_{50} value of ES cells is 0.56 mM and falls within the therapeutic range. Thus, ES cells show much more damage after VPA than CBZ within the therapeutic range [14]. To observe the cytotoxic and morphological effects of CBZ, ES cells and NIH-3T3 fibroblasts stained in the MTT were observed on day 5 of the cytotoxicity assay (Fig. 3, bottom). In both cell types, cell densities were reduced in a concentration-dependent manner. In the high dose CBZ group, NIH-3T3 fibroblasts showed strong indications of shrinkage or shape changes. In contrast, the ES cells contained many small, presumably undifferentiated cells, suggesting CBZ strongly inhibited differentiation. This observation is almost the same as that found using VPA [14].

Estimation of CBZ embryotoxicity with the mouse ES cell differentiation system

Of the various types of stem cell ES cells are one of the most valuable resources for development of *in vitro* model systems as they are capable of self-renewal and of differentiating into every mammalian cell lineage. Therefore, ES cells are one of the most suitable cell lines for analysis of the mutagenic, cytotoxic, and embryotoxic effects of chemicals [19]. In our previous study, we used the ES cell differentiation system to demonstrate the tissue-specific embryotoxic effects of VPA [14], one of the most teratogenic AEDs. In this study, we attempted to estimate the embryotoxic effects of CBZ, another type of teratogen, using the ES cell differentiation system. By analyzing the expression of tissue-specific genes and conducting immunocytochemical studies, we demonstrated that for endodermal and mesodermal cell lineages, the inhibitory potential of CBZ was less than that of VPA, and that the potential for neuronal differentiation induced by CBZ was lower than that by VPA. In addition, the cytotoxicity of CBZ for ES cells was much lower than that of VPA, and ES cells do not appear to be significantly affected by CBZ within the therapeutic range. These two drugs show clear differences in their *in vivo* embryotoxic effects. Our analyses using the ES cell assay system produced results that matched the *in vivo* differences and indicate that this assay

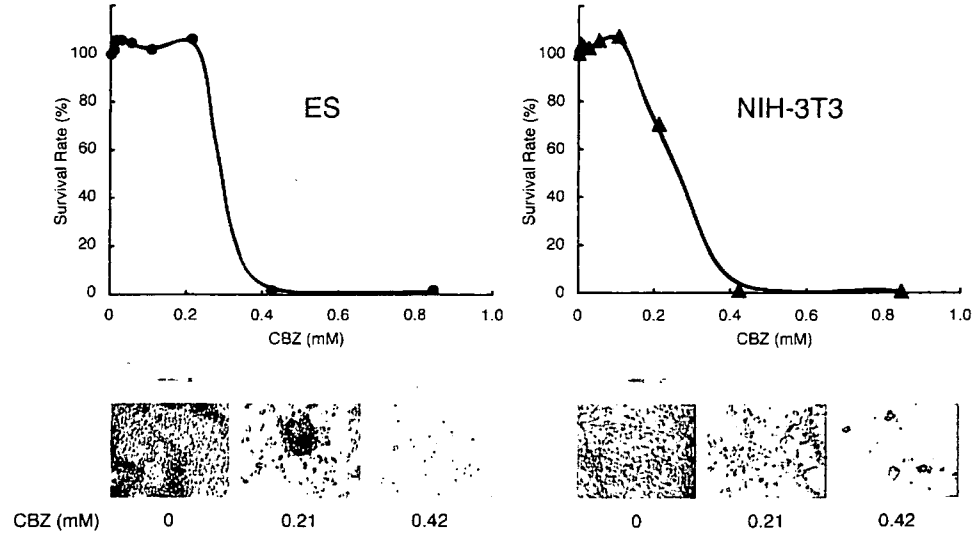

Fig. 3. Cytotoxicity assay on ES cells and NIH-3T3 fibroblasts. Cells on day 10 of the assay were stained with MTT and solubilized. The activity of the mitochondrial enzyme of living cells was examined. The violet color of the MTT formazan, which is the enzyme product, was measured at an absorbance of 520 nm. On day 5, cells were stained with MTT and examined with a Hoffman differential interference contrast microscope.

is very sensitive and is suitable for predicting *in vivo* embryotoxicity of therapeutic drugs.

Recently, CBZ as well as VPA have been reported to inhibit histone deacetylases (HDACs) [20–24]. HDAC inhibitors elevate histone acetylation levels and induce transcription of target genes, resulting in cell proliferation, differentiation, and apoptosis [25–27]. Recent reports suggest that the teratogenic effects of VPA are mediated by inhibition of HDACs [28,29]. It is possible that effects by CBZ may also be due to HDAC inhibition. Further studies on the embryonic toxicity of CBZ using our ES cell system should shed light not only on the relationship between CBZ and HDAC inhibition but also on how CBZ induces primitive differentiation.

Acknowledgments

This work was supported by research grants from the Scientific Fund of the Ministry of Education, Science, and Culture of Japan, the Ministry of Human Health and Welfare of Japan, and the Japan Health Science, and in part by grants from the Japan Spina Bifida and Hydrocephalus Research Foundation.

References

[1] M.A. Rogawski, P.J. Porter, Antiepileptic drugs: pharmacological mechanisms and clinical efficacy with consideration of promising developmental stage compounds, Pharmacol. Rev. 42 (1990) 223–286.
[2] F. Azarbayjani, Common mechanism of teratogenicity of antiepileptic drugs: drugs induced embryonic arrhythmia and hypoxia-reoxygenation damages. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy 253, Acta Universitatis Upsaliensis, Uppsala, 2001.
[3] S.H. Sindrup, T.S. Jensen, Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action, Pain 83 (1999) 389–400.
[4] F. Albani, R. Riva, A. Baruzzi, Carbamazepine clinical pharmacology: a review, Pharmacopsychiatry 28 (1995) 235–244.
[5] S.D. Shorvon, The epidemiology and treatment of chronic and refractory epilepsy, Epilepsia 28 (1996) S64–S70.
[6] K.L. Jones, R.V. Lacro, K.A. Johnson, J. Adams, Pattern of malformations in the children of women treated with carbamazepine during pregnancy, N. Engl. J. Med. 320 (1989) 1661–1666.
[7] T.H. Shepard, R.L. Brent, J.M. Friedman, K.L. Jones, R.K. Miller, C.A. Moore, J.E. Polifka, Update on new developments in the study of human teratogens, Teratology 65 (2002) 153–161.
[8] S. Matalon, S. Schechtman, G. Goldzweig, A. Ornoy, The teratogenic effect of carbamazepine: a meta-analysis of 1255 exposures, Reprod. Toxicol. 16 (2002) 9–17.
[9] B.F.D. Bourgeois, Valproic acid—clinical efficacy and use in epilepsy, in: R.H. Levy, R.H. Mattson, B.S. Meldrum, E. Perucca (Eds.), Antiepileptic Drugs, Lippincot Williams & Wilkins, Philadelphia, 2002, pp. 808–817.
[10] S.D. Silberstein, Clinical efficacy and use in other neurological disorders, in: R.H. Levy, R.H. Mattson, B.S. Meldrum, E. Perucca (Eds.), Antiepileptic Drugs, Lippincot Williams & Wilkins, Philadelphia, 2002, pp. 818–827.
[11] H. Nau, R.S. Hauck, K. Ehlers, Valproic acid-induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms, Pharmacol. Toxicol. 69 (1991) 310–321.
[12] H. Nau, Valproic acid-induced neural tube defects, Ciba Found. Symp. 181 (1994) 144–160.
[13] A. Ornoy, Neuroteratogens in man: an overview with special emphasis on the teratogenicity of antiepileptic drugs in pregnancy, Reprod. Toxicol. 22 (2006) 214–226.
[14] M. Murabe, J. Yamauchi, Y. Fujiwara, M. Hiroyama, A. Sanbe, A. Tanoue, A novel embryotoxic estimation method of VPA using ES cells differentiation system, Biochem. Biophys. Res. Commun. 352 (2007) 164–169.
[15] A.M. Wobus, K. Guan, H.T. Yang, K. Boheler, In embryonic stem cells: methods and protocols, in: K. Turksen (Ed.), Methods in Molecular Biology, Humana, Totowa, NJ, 2002, pp. 127–156.
[16] H. Spielmann, I. Pohl, B. Dröing, M. Liebsch, F. Moldenhauer, The embryonic stem cell test, *in vitro* embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblast and embryonic stem cells, In Vitro Toxicol. 10 (1997) 119–127.
[17] T. Mosman, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[18] R.M. Cabrera, D.S. Hill, A.J. Etheredge, R.H. Finnell, Investigations into the etiology of neural tube defects, Birth Defects Res. Part C 72 (2005) 330–344.
[19] J. Rohwedel, K. Guan, H.T. Yang, A.M. Wobus, Embryonic stem cells as an *in vitro* model for mutagenicity, cytotoxicity and embryotoxicity studies: present state and future, Toxicol. In Vitro 15 (2001) 741–753.
[20] C.J. Phiel, F. Zhang, E.Y. Huang, M.G. Guenther, M.A. Lazar, P.S. Klein, Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen, J. Biol. Chem. 276 (2001) 36734–36741.
[21] M. Gottlicher, S. Minucci, P. Zhu, O.H. Kramer, H.A. Schimpf, S. Giavara, J.P. Sleeman, F. Lo Coco, C. Nervi, P.G. Pelicci, T. Heinzel, Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells, EMBO J. 20 (2001) 6969–6978.
[22] L. Tremolizzo, G. Carboni, W.B. Ruzicka, C.P. Mitchell, I. Sugaya, P. Tueting, R. Sharma, D.R. Grayson, E. Costa, A. Guidotti, An epigenetic mouse model for molecular and behavioral neuropathologies related to schizophrenia vulnerability, Proc. Natl. Acad. Sci. USA 99 (2002) 17095–17100.
[23] E. Yildirim, Z. Zhang, T. Uz, C.Q. Chen, R. Manev, H. Manev, Valproate administration to mice increases histone acetylation and 5-lipoxygenase content in the hippocampus, Neurosci. Lett. 345 (2003) 141–143.
[24] A.S. Beutler, S.L. Li, R. Nicol, M.J. Walsh, Carbamazepine is an inhibitor of histone deacetylases, Life Sci. 76 (2005) 3107–3115.
[25] B.D. Strahl, C.D. Allis, The language of covalent histone modifications, Nature 403 (2000) 41–45.
[26] P.A. Marks, T. Miller, V.M. Richon, Histone deacetylases, Curr. Opin. Pharmacol. 3 (2003) 344–351.
[27] L. Tou, Q. Liu, R.A. Shivdasani, Regulation of mammalian epithelial differentiation and intestine development by class I histone deacetylases, Mol. Cell. Biol. 24 (2004) 3132–3139.
[28] N. Gurvich, M.G. Berman, B.S. Wittner, R.C. Gentleman, P.S. Klein, J.B.A. Green, Association of valproate-induced teratogenesis with histone deacetylase inhibition in vivo, FASEB J. 19 (2005) 1166–1168.
[29] D. Eikel, A. Lampen, H. Nau, Teratogenic effects by inhibition of histone deacetylases: evidence from quantitative structure–activity relationships of 20 valproic acid derivatives, Chem. Res. Toxicol. 19 (2006) 272–278.

Available online at www.sciencedirect.com
ScienceDirect
Reproductive Toxicology 24 (2007) 178–198
Reproductive Toxicology
www.elsevier.com/locate/reprotox

Review

In vitro molecular mechanisms of bisphenol A action☆

Yelena B. Wetherill[a,b], Benson T. Akingbemi[c], Jun Kanno[d], John A. McLachlan[e], Angel Nadal[f], Carlos Sonnenschein[g], Cheryl S. Watson[h], R. Thomas Zoeller[i], Scott M. Belcher[j,*]

[a] *Department of Environmental Health, Harvard School of Public Health, Boston, MA 02115, United States*
[b] *Department of Epidemiology, Harvard School of Public Health, Boston, MA 02115, United States*
[c] *Department of Anatomy, Physiology and Pharmacology, Auburn University, AL 36849, United States*
[d] *Division of Cellular and Molecular Toxicology, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[e] *Department of Pharmacology and Environmental Endocrinology Lab, Center for Bioenvironmental Research, Tulane University, New Orleans, LA 70112, United States*
[f] *Instituto de Bioingeniería, Universidad Miguel Hernández de Elche, Elche 03202, Alicante, Spain*
[g] *Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, MA 02111, United States*
[h] *Biochemisty and Molecular Biology Department, University of Texas Medical Branch, Galveston, TX 77555, United States*
[i] *Laboratory of Molecular and Cellular Neurobiology, University of Massachusetts Amherst, MA 01003, United States*
[j] *University of Cincinnati College of Medicine, Department of Pharmacology and Cell Biophysics, Cincinnati, OH 45267, United States*

Received 12 April 2007; accepted 18 May 2007
Available online 29 May 2007

Abstract

Bisphenol A (BPA, 2,2-bis (4-hydroxyphenyl) propane; CAS# 80-05-7) is a chemical used primarily in the manufacture of polycarbonate plastic, epoxy resins and as a non-polymer additive to other plastics. Recent evidence has demonstrated that human and wildlife populations are exposed to levels of BPA which cause adverse reproductive and developmental effects in a number of different wildlife species and laboratory animal models. However, there are major uncertainties surrounding the spectrum of BPA's mechanisms of action, the tissue-specific impacts of exposures, and the critical windows of susceptibility during which target tissues are sensitive to BPA exposures. As a foundation to address some of those uncertainties, this review was prepared by the "*In vitro*" expert sub-panel assembled during the "*Bisphenol A: An Examination of the Relevance of Ecological, In vitro and Laboratory Animal Studies for Assessing Risks to Human Health*" workshop held in Chapel Hill, NC, Nov 28-29, 2006. The specific charge of this expert panel was to review and assess the strength of the published literature pertaining to the mechanisms of BPA action.

Abbreviations: 4-*tert*-OP, 4-tertiary octylphenol; 17α-EE, 17alpha-ethinyl estradiol; AR, androgen receptor; ARE, androgen response element; BADGE, bisphenol A diglycidyl ether; BFDGE, bisphenol F diglycidyl ether; BP-3, *p,p'*-dihydroxybenzphenone; BP-4, 2,2-bis(*m*-methyl-*p*-hydroxyphenyl)propane; BP-5, 2,2-bis(*p*-hydroxyphenyl) perfluoropropane; BPA, 2,2-bis(4-hydroxyphenyl) propane; BPA-OMe, 2,2-bis(4-methoxyphenyl)propane; BPE, 1,1-bis(4-hydroxyphenyl)ethane; BPF, 4,4'-methylenebisphenol; BPM, 4,4'-(1,3-phenylenedisopropylidene)bisphenol; BPP, 4,4'-(1,4-phenylenedisopropylidene)phenol; BPS, 4,4'-sulfonyldiphenol; BPZ, 4,4'-cyclohexylidenebisphenol; DDE, *o,p'*-dichlorodiphenylethylene; *o,p'*-DDT, 1,1,1-trichloro-2-[*o*-chlorophenyl]-2-[*p*-chlorophenyl]ethane; DEHP, bis(2-ethylhexyl)phthalate; DES, diethylstilbesterol; DHT, dihydrotestosterone; DMSO, dimethyl sulfoxide; E2, 17beta-estradiol; EDC, endocrine disrupting chemical; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; ERE, estrogen response element; ETOH, ethanol; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; GH, growth hormone; HBSS, Hank's buffered saline solutions; h, hour; HRP, horseradish peroxidase; inos, inducible nitric oxide synthase; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; min, minute; NO, nitric oxide; NP, nonylphenol; OP, octylphenol; PCB, polychlorinated biphenyl; PMA, phorbol 12-myristate 13-acetate; PR, progesterone receptor; PSA, prostrate specific antigen; RIA, radio-immuno assay; RT-PCR, reverse transcription-polymerase chain reaction; RXR, retinoid X receptor; s, second; TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; T3, L-3,3',5-triiodothyronine; T4, 3,3',5,5'-tetraiodo-L-thyronine; TR, thyroid hormone receptor.

☆ SMB is supported by the NIH grants RO1-ES015145 and the University of Cincinnati Center for Environmental Genetics (P30-ES06096). YBW is supported by the NIEHS Kirschstein-National Research Service Award (T32 ES07069-26).

* Corresponding author at: Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Albert Sabin Way, P.O. Box 670575, Cincinnati, OH 45267-0575, Untied States. Tel.: +1 513 558 1721; fax: +1 513 558 4329.
E-mail address: scott.belcher@uc.edu (S.M. Belcher).

0890-6238/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.reprotox.2007.05.010

The resulting document is a detailed review of published studies that have focused on the mechanistic basis of BPA action in diverse experimental models and an assessment of the strength of the evidence regarding the published BPA research.

Keywords: Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl) propane; Endocrine disruption; Endocrine disrupting chemical (EDC); Rapid signaling; Estrogen receptor; Androgen receptor; Thyroid receptor; Thyroid hormone; GPR30; Non-classical membrane estrogen receptor; Immune system; Allergic resonse; *In vitro* mechanisms; Cell specificity; Expert panel review

Contents

1. Introduction

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl) propane; CAS# 80-05-7) is a chemical used primarily in the manufacture of polycarbonate plastic, epoxy resins and as a non-polymer additive to other plastics. Because of BPA's extensive use in the manufacture of consumer goods and products, including polycarbonate food containers and utensils, dental sealants, protective coatings, some flame retardants, and water supply pipes, there is a widespread and well-documented human exposure to BPA [1,2]. Recent discoveries regarding the environmental distribution and presence of BPA in humans and wildlife have generated persistent scientific, regulatory, and public interest in assessing the potential health risks associated with BPA exposure. The detection of adverse health effects in a number of laboratory animal models upon exposure to environmentally relevant doses of BPA, as well as potential effects on human reproduction and development, have fueled additional concern. While the exposure of wildlife species and humans to BPA has been increasingly reported in the literature, there are research gaps regarding its molecular mechanisms of action, the tissue-specific impacts of exposure, and knowledge of the critical windows of susceptibility, during which target tissues are especially sensitive to BPA.

The lack of an integrated and systemic understanding of BPA's endocrine disruptive actions has considerably complicated risk assessment efforts and safety recommendations by regulatory agencies. Current limitations in understanding the global consequences of BPA exposures include incomplete understanding of the cell/tissue specific actions and effects, a limited understanding of the receptor systems and signaling cascades through which BPA acts, and understanding when these receptors and signaling systems are present and active in target cells at different critical times of sensitivity during the entire life-span.

2. Overview

This white paper was prepared for the *in vitro* expert subpanel assembled during the "Bisphenol A: An Examination of the Relevance of Ecological, *In vitro* and Laboratory Animal Studies for Assessing Risks to Human Health" workshop at the NIEHS. The specific charge of this expert panel was to review and assess the strength of the published literature pertaining to the mechanisms of BPA action. Particular attention was paid to studies employing *in vitro* models and the results, findings and conclusions from those studies were integrated into the broader biological/physiologic context of BPA action. Thus, the goal of

the white paper was to review in detail peer-reviewed published studies that have focused on the mechanistic basis of BPA action in diverse experimental models.

3. Definition of endocrine disruptor

The U.S.-Environmental Protraction Agency (EPA) has defined an environmental endocrine disruptor or endocrine disrupting chemical (EDC) as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" [3]. This definition is not limited to endocrine disrupting effects exclusive of the estrogen system. Rather, endocrine disruption encompasses effects on other endocrine systems including effects mediated by androgens, thyroid hormone, prolactin, and insulin, among others.

4. Definition of "low dose"

The primary focus of this review is on *in vitro* studies that investigate the molecular mechanisms of BPA action. However, full consideration of BPA's mode of action cannot rely solely on *in vitro* studies performed at a range of concentrations. It is critically important to consider experimental findings from numerous *in vivo* studies investigating BPA actions in animals at a physiologically relevant exposure dose, also known as a "low-dose". Thus, a low-dose effect was determined based on the U.S.-EPA criterion for low-dose effects of EDCs as effects observed at concentrations below those used for traditional toxicological studies. Levels below the current lowest observed effect level (LOAEL) of 50 μg/kg/day were considered as low dose for *in vivo* studies. For *in vitro* cell or organ culture studies, estimates of circulating levels of BPA at the LOAEL cut-off have defined an equivalent low dose concentration as <50 ng/mL or $<2.19 \times 10^{-7}$ M [4]. Based on experimental designs of most studies, we elected to consider an alternative and more conservative definition of low-dose for *in vitro* BPA effects as $\leq 1 \times 10^{-7}$ M.

5. Definition of estrogen

When considering the mechanisms of BPA action, it was considered critical to determine whether the molecular and physiological EDC effects of BPA could be fully explained by its ability to act as an agonist or antagonist of endogenous estrogens in target cells, or whether the molecular and physiological EDC effects of BPA extend beyond the molecular, subcellular and/or cellular mechanism of estrogens' actions. According to the Merriam-Webster Medical Dictionary of the U.S. National Library of Medicine, (http://www.nlm.nih.gov/medlineplus/mplusdictionary.html) estrogen is defined as: any of various natural steroids (as estradiol) that are formed from androgen precursors, that are secreted chiefly by the ovaries, placenta, adipose tissue, and testes, and that stimulate the development of female secondary sex characteristics and promote the growth and maintenance of the female reproductive system; also: any of various synthetic or semisynthetic steroids (as ethinyl estradiol) that mimic the physiological effect of natural estrogens.

In this review, "estrogen" is used as a class name for endogenous estrogenic compounds, and "estradiol" or "17β-estradiol" will be used more specifically in reference to 1,3,5(10)-estratrien-3,17β-diol.

6. Mechanisms of BPA action

Diverse biological effects have been attributed to low-dose environmental BPA exposure in a variety of tissues. Molecular studies employing an array of *in vitro* models have revealed a variety of molecular pathways through which BPA may elicit cellular responses. As a prototypical non-steroidal estrogen, BPA interferes with the activity of endogenous estrogens (e.g. 17β-estradiol) by disrupting the proper activity of the estrogen nuclear hormone receptors in a diverse set of target tissues [5–21]. However, the primary endocrine disrupting activities of BPA extend beyond its ability to mimic, enhance or inhibit the activity of endogenous estrogens and/or disrupt estrogen nuclear hormone receptor action, and include the following: effects upon the androgen systems [22–29]; disruption of thyroid hormone function [30–40]; diverse influences on development, differentiation and function of the central nervous system [40–46]; and influences on the immune system [21,47–65]. There is additional clear evidence for BPA to directly impact intracellular signal transduction pathways through mechanisms independent of the transactivational activity of nuclear hormone receptors [43,46,66–79]. Secondary metabolic and pharmacokinetic actions of BPA that impact its bioavailability and bioavailability of endogenous steroid hormones have also been described. Those secondary effects include modification of cytochrome P450 enzyme expression and activity [80–83] and alterations of serum hormone binding protein expression and interactions [84–87]. Recent studies have also begun to highlight the potential for transmission of early exposures to physiological expression later in life and across generations via epigenetic mechanisms such as methylation-meditated promoter silencing [88–90]. Table 1 summarizes the results from published studies that were used in the preparation of this review. Clearly, BPA is an endocrine disrupting compound or EDC in the broad sense of the definition. However, because of the pleiotropic mechanisms of BPA action, narrowly defining BPA as a selective estrogen receptor modulator [SERM; a class of pharmacological compounds (such as raloxifene or tamoxifen) that bind nuclear estrogen receptors and act as estrogen agonists in some tissues and estrogen antagonists in other tissues], or exclusively as an environmental estrogen, is inaccurate. The use of these self-limiting terms only partially describes the extent of BPA's endocrine disrupting and other biological activities.

7. Nuclear estrogen receptors, coregulators, and the estrogen-response element (ERE)

It is well established that BPA can exert some of its effects by binding at the nuclear steroid receptors ERα and ERβ to induce estrogenic signals that modify estrogen-responsive gene expression. Mechanisms for ER-mediated gene regulation are

Table 1
Published papers reporting physiological effects of BPA *in vitro*

Reference	Species	Model	Endpoints	Exposure; vehicle	Exposure time	LOEC (M) (*$P < 0.05$)	Doses tested (M) (*$P < 0.05$)	Inhibitors	Other chemicals tested
Adipose models									
Masuno et al. 2005 [167]	Mice	3T3-L1 (preadipocyte)	Adipocyte differentiation	Culture medium, Not indicated	6 days	2×10^{-5}*	4×10^{-6}, 2×10^{-5}*, 4×10^{-5}*, 8×10^{-5}*	LY294002	BPA-derivatives; NP; OP
Masuno et al. 2002 [168]	Mice	3T3-L1 (preadipocyte)	Adipocyte differentiation	Culture medium, Not indicated	11 days	2×10^{-6}*	2×10^{-6}*, 10^{-5}*, 2×10^{-5}*		BPA-derivatives
Bone models									
Kanno et al. 2004 [169]	Mice	MC3T3-E1 (C57BL/6) osteoblast-like	Cell proliferation, Differentiation (ALP activity), Ca^{2+} content	Culture medium, ETOH	3–4 days	10^{-4}*, 10^{-6}*, 10^{-6}*	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}*, 10^{-5}*, 10^{-4}*		E2; coumestrol; genistein; daidzein; NP; DEHP
Breast cancer models									
Buterin et al. 2006 [101]	Human	T47D/Luc, MCF7	ERE-promoter activation, Cell proliferation	Culture medium, DMSO	3–24 h, 24 h	10^{-7} (24 h)	10^{-13} to 10^{-5}, 10^{-5}		E2; genistein; PCB54; PCB126
Cappelletti et al. 2003 [170]	Human	T47D, BT20	Cell growth, ER, PR mRNA (RT-PCR)	Culture medium, Not indicated	6 days, 10, 24, 48 h	10^{-6}*, 10^{-8}*	10^{-8}, 10^{-7}, 10^{-6}*, 10^{-5}*		E2; 4-*tert*-OP; 2-OH-biphenol
Inadera et al. 2000 [60]	Human	MCF7	IL-1 induced MCP-1 expression	Culture media, <0.1% ETOH	24 h	5×10^{-7}*	10^{-7}, 5×10^{-7}*, 10^{-6}*	ICI 182,780	E2; NP
Iso et al. 2006 [171]	Human	MCF7, MDA-MB-231	DNA damage (comet tail length assay)	Culture medium, <0.1% ETOH	1, 3, 24 h	10^{-6}*	10^{-8}, 10^{-6}*, 10^{-4}*	ICI 182,780	E2
Olsen et al. 2003 [15]	Human	MCF7	Cell proliferation, Estrogen-responsive gene expression	Culture medium, ≤0.2% ETOH	6 days, 3 days	5×10^{-7}*	10^{-8}, 10^{-7}, 5×10^{-7}*, 10^{-6}*, 10^{-5}*	4-OHTamoxifen; ICI 182,780	E2; tetra-Cl-BPA; tetra-Br-BPA; T3; T4; 4-OH-biphenol; 4,4-OH biphenol
Recchia et al. 2004 [9]	Human	MCF7, T47D	Cell proliferation, ERα-expression & nuclear localization, ERE-luciferase reporter	Culture medium, Not indicated	6 days, 2 h	10^{-6}*	10^{-6}*	4-OH Tamoxifen	4-NP
Samuelsen et al. 2001 [142]	Human	MCF7, T47D	Cell proliferation, PR, pS2-expression	Culture medium, ≤0.1% ETOH	6 days	10^{-7}	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}		E2; Br-BPA derivatives
Singleton et al. 2006 [98]	Human, *S. cerevisiae*	MCF7-C4-12 (HA-ERα) Ishikawa (endometrial)	Differential mRNA expression, ERE-LacZ	Culture media, ETOH	3 h, 24 h	10^{-6}, 10^{-7}	10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}		E2
Vivacqua et al. 2003 [10]	Human	MCF7, MCF7SH	ERE-luciferase, ERα, pS2 expression, Cell growth	Culture medium, Not indicated	20–24, 6 days	10^{-9}	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}*, 10^{-6}, 10^{-5}	4-OH Tamoxifen	E2; 4-NP
Walsh et al. 2005 [74]	Human	MCF7, MDA-MB-231, SKBR-3	Intracellular Ca^{2+}	Kreb's solution, ≤0.001% methanol	0–15 min	10^{-10}*	10^{-10}*, 10^{-9}*, 10^{-8}*, 10^{-7}*	ICI 182,780	*o,p'*-DDT; DES; 4-*tert*-OP
Embryonic/development models									
Iwamuro et al. 2003 [146]	*Xenopus laevis*	Tadpole tail organ culture	Tail length regression (also see *in vivo*)	Culture medium, ETOH	1–4 days	10^{-6}*	10^{-6}*, 10^{-5}*, ($\pm 10^{-7}$T3)		E2
Iwamuro et al. 2006 [32]	*Xenopus laevis*	Tadpole tail organ culture	Tail length regression, TRα, TRβ, RXRγ, mRNA expression (RT-PCR)	Culture medium, ETOH	1–5 days	10^{-7}*	10^{-7}*, 10^{-6}*, 10^{-5}*, ($\pm 10^{-7}$ T3)		
Tsutsui et al. 1998 [172]	Hamster (Syrian)	Primary embryo cells	Cell growth, morphological chromosomal aneuploidy, DNA adduct formation	Culture medium, 0.6% DMSO	24, 48, 72 h	5×10^{-5}*	5×10^{-5}*, 10^{-4}*, 2×10^{-5}*		
Tsutsui et al. 2000 [173]	Hamster (Syrian)	Primary embryo cells	Cell growth, morphological, DNA adduct formation, aneuploidy	Culture medium, 0.6% DMSO	24, 48, 72 h	10^{-4}*	5×10^{-5}*, 10^{-4}*, 2×10^{-5}*		BPF; BP-3; BP-4; BP5
Endothelium models									
Lee et al. 2003 [25]	Human	Female primary endothelial cells	Cell proliferation	Culture medium, Not indicated	24, 48, 72 h	10^{-6}*	10^{-8}, 10^{-6}*, 10^{-4}*		Aroclor 1254
Female reproductive tissue models									
Jin et al. 2005 [174]	Human	BeWo (b30) placental trophoblast	Calcein AM efflux	HBSS 25 mM glucose, ETOH 0.1%	48 h	10^{-7}*	10^{-7}*, 10^{-5}*, 5×10^{-5}*		E2
Xu et al. 2002 [112]	Mice B6C3F1	Primary granulosa cells	Cell viability/apoptosis	Culture medium, 0.1% ETOH	72 h	10^{-10}*	10^{-13}, 10^{-10}*, 10^{-7}*, 10^{-4}*		
Immue system models									
Alizadeh et al. 2006 [47]	Mice BALB/c	Primary splenocytes; Sex not specified	Cytokine production, (IL-4;-12; IFN-γ ELISA)	*In vivo* IP-injection, corn oil	8 days	100 mg/kg every other days	100 mg/kg for IL-4*; IFN-γ*		
Canesi et al. 2004 [75]	Mussels	Primary hemocytes	Lysosomal membrane stability, ERK2, p38, STAT3, STAT5 phosphorylation	Artificial sea water/serum, ETOH	15–110 min, 5–30 min	2.5×10^{-5}*, 2.5×10^{-5}*	2.5×10^{-5} 2.5×10^{-4}, 2.5×10^{-3}*,	4-OHTamoxifen; SB203580; PD98059; wortmannin; GF109203X	DES; NP

Table 1 (*Continued*)

Reference	Species	Model	Endpoints	Exposure; vehicle	Exposure time	LOEC (M) (*$P < 0.05$)	Doses tested (M) (*$P < 0.05$)	Inhibitors	Other chemicals tested
Goto et al. 2004 [64]	Mice BALB/c	Female primary splenocytes	Cell proliferation	Culture medium, 0.156% DMSO	72 h	2×10^{-5}*	2.5×10^{-6}, 5×10^{-6}, 1×10^{-5}, 2×10^{-5}*		
Han et al. 2002 [63]	Mice BALB/c	Male primary splenocytes	Immunoglobulin production (ELISA)	Culture medium, <0.1% ETOH	7 days	10^{-8} (IgE; IgM)	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-3}		E2; tamoxifen; DES; diadzein genistein quercetin; luteolin
Hong et al. 2004 [62]	Mice BALB/c	RAW 264 (macrophage), Female Abdominal macrophages	LPS-induced TNF-α production, NO-production	Culture medium DMSO, DMSO/corn oil	4 h	nd	4.4×10^{-5}* (NO only), NS		Various EDCs
Kim et al. 2003 [48]	Mice	RAW 264.7 (macrophage), Female Abdominal macrophages	TNFα; NO production (total nitrite), iNOS expression (RT-PCR)	Culture medium (± LPS), <0.1% DMSO	6, 24 h	10^{-5}*	10^{-6}, 10^{-5}*, 5×10^{-5}*, * vs. LPS control	ICI 182,780	E2
Lee et al. 2003 [61]	Mice BALB/c	Female primary lymph node cells, hybridoma cells (3.155, GK1.5, EL4)	PMA/KLH-primed IL4-production	Culture medium, Not indicated	2, 4 days	10^{-5}*	10^{-7}, 10^{-6}, 10^{-5}*, 5×10^{-5}*, 10^{-4}*	Nitrendipine; BATA-AM; thapsigargin; FK506	NP
Ndebele et al. 2004 [49]	Human	CD4+ Jurkat T cell line	IL-2 production (ELISA; RT-PCR)	Culture medium	48 h	ns	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}		Coumesterol; TCDD; DDT
Reistad et al. 2005 [160]	Human	Primary neutrophil granulocytes	Reactive oxygen species, Intracellular Ca^{2+} (secondary endpoints)	HEPES-buffered salt solution, ≤0.1% ETOH	60 min, 250 s	2×10^{-6}*	2×10^{-6}*, 6×10^{-6}*, 1.2×10^{-5}*, 2×10^{-5}*		4-Br-BPA
Sakabe et al. 1999 [50]	Mice	Primary thymus epithelial cells	Thymosin-α1 suppression (ELISA; HPLC)	Culture medium, Not indicated	7 days	3×10^{-6}*	3×10^{-8}, 3×10^{-7}, 3×10^{-6}*, 3×10^{-5}*3×10^{4}*		E2; genistein; coumesterol; α−zeralanol; progesterone; cholesterol
Sawai et al. 2003 [51]	Mice C57BL/6;	Primary splenic mononuclear cells	ConA induced IFN-γ secretion (ELISA)	Culture medium, Not indicated	24 h	5×10^{-8}*	5×10^{-10}, 5×10^{-8}*, 5×10^{-6}*		
Watanabe et al. 2003 [53]	Human	HL-60	Neutrophilic differentiation, O_2^- production, CD18 expression	Culture medium, DMSO	6 days	10^{-10}*	10^{-12}, 10^{-10}, 10^{-9}*, 10^{-8}	4-OH Tamoxifen	
Liver models									
Jeong et al. 2000 [83]	Mice	Hepa-1c1c7 (hepatoma)	Cyp1a-1 activity, Cyp1a-1 mRNA expression (RT-PCR)	Culture medium, DMSO <0.2%	18 h, 6 h	10^{-5}*	10^{-6}, 10^{-5}*, 5×10^{-5}*	4-OH Tamoxifen	TCDD
Kubo et al. 2004 [78]	Human	Hep3B (hepatoma)	Inhibition of hypoxic response, Erythropoietin, HIF-1a expression	Culture medium, Not indicated	6 h	10^{-4}	5×10^{-5}, 10^{-4}, 5×10^{-4}	CoCl; Mg132	BPA-OMe; BPE; BPF
Male reproductive tissue models									
Fiorini et al. 2004 [136]	Rat	SerW3 (Sertoli cells testis)	Qualitative junctional protein expression	Culture medium, DMSO <0.1%	24 h	4.5×10^{-5}	4.5×10^{-5}		BPA-methacrylate; *o,p'*-DDT
Iida et al. 2003 [175]	Rat Wistar	Primary Sertoli cells	Viability/apoptosis	Culture media	<12–48 h	1.5×10^{-4}*	5×10^{-5}, 10^{-4}, 1.5×10^{-4}*, 2×10^{-4}*, 3×10^{-4}*		
Lee et al. 2003 [29]	*S. cerevisiae*, Human, Mice	Hela, HepG2, 15p-1 (Sertoli cell line)	2-hybrid AR/ASC1, GFP-AR translocation, Inhibition of ARE-transcription (luciferase)	Culture medium, ETOH	3 h, 24 h, 24 h	10^{-6}	10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}		E2; NP; cyproterone
Nikula et al. 1999 [135]	Mice	mLTC-1 (Leydig tumor cell line)	cAMP, progesterone production	Culture medium, ETOH	48 h	10^{-7}*	10^{-7}, 10^{-6}*, 10^{-5}*, 10^{-4}*		E2; *tert*-OP; OP; DES
Song et al. 2002 [137]	Mice	K28 (Leydig tumor cell line)	Nur77 expression S, Steroidogenic function	Culture media, 0.1% ETOH	30 min −24 h	10^{-8}	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}	H89; PD98059; GFX; wortmannin	BPE; BPF; BPM; BPP; BPS; BPZ
Tabuchi et al. 2006 [176]	Mice	TTE3 Sertoli cells (testis)	mRNA expression	Culture media, 0.1% DMSO	3, 6, 12 h	2×10^{-4}*	2×10^{-4}*		
Wetherill et al. 2002 [26]	Human	LNCaP (prostate)	Cell proliferation, AR-translocation	Culture medium, 0.1% ETOH	72 h, 2, 7 h	10^{-9}*, 10^{-10}*	10^{-10}, 10^{-9}*,10^{-8}, 10^{-7}	Bicalutamide	DHT
Wetherill et al. 2005 [28]	Human	LNCaP (prostate)	PSA, AR expression, AR transactivation	Culture medium, 0.1% ETOH	48 h	10^{-9}	10^{-9}, 10^{-5}	Bicalutamide	DHT
Satoh et al. 2004 [12]	Hamster	CHO-K1 (ARE—luciferase)	AR/ARE—luc expression (± DHT), Cell proliferation	Culture medium, DMSO, ≤0.1% ETOH	16–24 h	10^{-6}, 10^{-7}	10^{-11}, 10^{-10}, 10^{-9}, 10^{-7}, 10^{-7}, 10^{-5}		BADGE; BADGE-2H_2O; BADGE-2HCl; BPF; BFDGE; BFDGE-2H_2O; BFDGE-2HCl; Cyproterone; Mibolerone
Nervous system models									
Miyatake et al. 2006 [44]	Mice ICR	Primary culture midbrain astrocytes & neuron/glia	GFAP/Neu-N immunoreactivity, [Ca^{2+}]I	Culture medium, Not indicated	24 h	10^{-13}*	10^{-14}, 10^{-13}*, 10^{-12}*, 10^{-11}*, 10^{-10}, 10^{-10}, 10^{-10}, 10^{-7}*, 10^{-6}*	ICI 182,780; mifepristone; flutamide	E2

Table 1 (*Continued*)

Reference	Species	Model	Endpoints	Exposure; vehicle	Exposure time	LOEC (M) (*$P < 0.05$)	Doses tested (M) (*$P < 0.05$)	Inhibitors	Other chemicals tested
Seiwa et al. 2004 [177]	Mice ICR	Primary oligodendrocyte precursor cells	T3-induced differentiation	Culture media, Not indicated	2 days	10^{-5}	10^{-9}, 10^{-7}, 10^{-6}, 0^{-5}		
Sato et al. 2002 [178]	Rat Wistar	Hippocampus organotypic primary culture	Glutamate-induced cell death (CA3)	Culture medium	24 h	10^{-12}*	10^{-12}*	ICI 182,780;	E2; 17α-EE; DES; p-NP
							10^{-9}*	4-OH Tamoxifen	
							10^{-6}*		
			NMDA-receptor expression, Spine & mossy fiber density	≤0.1% ETOH			10^{-4}*		
Yamaguchi et al. 2006 [40]	Mice	SFME cells	LIF/BMP-2 induced astrocyte differentiation	Culture medium, Not indicated	48 h	4.4×10^{-12}*	4.4×10^{-13}, 4.4×10^{-12}*, 4.4×10^{-11}*, 4.4×10^{-10}*, 4.4×10^{-9}, 4.4×10^{-8}, 4.4×10^{-7}, 4.4×10^{-6}	4-OH Tamoxifen	
Yoneda et al. 2003 [76]	Rat	PC12 (adrenal)	Dopamine secretion	Culture medium, ≤0.1% DMSO	10 min	2.5×10^{-6}*	2.5×10^{-6}*, 5×10^{-6}*, 1×10^{-5}*, 1.5×10^{-5}*	Diltiazem; clorgyin; pargyline; ω-agatoxin; ω-conotoxin; H7; H-89; ryanodine; ruthenium red; Rp-cAMPS; GDPβs,	
Zsarnovszky et al. 2005 [46]	Rat Sprague-Dawley	Developing cerebellar neurons (male & female)	ERK1/2 phosphorylation	Culture medium, artificial cerebrospinal fluid, DMSO	5 min	10^{-12}*	10^{-12}*, 10^{-11}*, 10^{-10}*, 10^{-9}, 10^{-8}, 10^{-7}*, 10^{-6}*, 10^{-4}	U0128	E2
Pancreatic models									
Adachi et al. 2005 [21]	Rat Wistar	Male; primary pancreatic islets	Insulin release-RIA	Krebs-Ringer bicarbonate buffer −16.7 mM glucose	60 min, 24 h	nd, 4.4×10^{-8}*	4.4×10^{-10}, 4.4×10^{-9}, 4.4×10^{-8}*, 4.4×10^{-8}*, 4.4×10^{-6}	Actinomycin-D; ICI 182,780	E2; NP
Alonso-Magdalena et al. 2005 [66]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Intracellular Ca^{2+}	Modified Ringers—5 or 0.5 mM glucose, DMSO	5 min	10^{-9}*	10^{-9}*	ICI 182,780; KT-5823; pertussis toxin; ODQ; L-NAME	E2; E2-HRP; DES; DDT
Alonso-Magdalena et al. 2006 [67]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Insulin release-RIA; insulin content	*In vivo* sc injection; tocopherol-stripped corn oil		10 μg/kg/day	10; 100 μg/kg/day	ICI 182,780	E2
Nadal et al. 2000 [70]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Fluo-3 AM $[Ca^{2+}]i$	Perfusion buffer −8 mM glucose	4–8 min	10^{-10}	10^{-10}, 10^{-11}, 10^{-9}, 10^{-7}		E2; E2-HRP; DES; *o,p'*-DDT
Quesada et al. 2002 [73]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Intracellular Ca^{2+}, Phospho-CREB-IR	Profusion buffer	0–15 min	10^{-9}	10^{-9}	ICI 182,780	E2; E2-HRP
Pituitary models									
Bulayeva et al. 2004 [69]	Rat	GH3/B6/F10 (ERα-positive)	ERK1/2 phosphorylation immunoreactivy	Culture medium; ETOH	3–30 min	nd	10^{-9}		E2; NP; endosulfan; DDE; dieldrin; coumesterol
Ghisari et al. 2005 [30]	Rat	GH3	Cell proliferation (T-screen)	Culture medium	6 days	10^{-7}*	10^{-8}, 10^{-7}*, 10^{-6}*, 10^{-5}*	ICI 182,780	E2; T3; BPA-methacrylate; 4-OP; 4-n-NP; iprodion; prochloraz; chlorpyrifos; 4-OH-PCB 106; 4-OH-PCB 69; 4-OH-PCB121
Kitamura et al. 2002 [33]	Rat	GH3	Cell proliferation	Culture medium, ETOH	7 days	10^{-6}*	10^{-7}, 10^{-6}*10^{-5}*	4-OH Tamoxifen	T3; E2; TBBPA; TCBPA
Kitamura et al. 2005 [35]	Human, Mice, Rat	MCF7, NIH3T3, GH3	ERE_3-luciferase, ARE-luciferase, GH-induction	Culture medium, ETOH	24 h, 24 h, 2 days	10^{-7}*, 10^{-6}*, NS	10^{-8}, 10^{-7}*, 10^{-6}*, 10^{-5}*, 10^{-4}*	4-OHTamoxifen	E2; various BPA-related compounds
Steinmetz et al. 1997 [157]	Rat	GH3	Prolactin release, Cell proliferation, Prolactin-luciferase	Culture media, ≤0.001% ETOH	1–8 days, 8, 24 h	10^{-6}, 10^{-9}	10^{-6}, 10^{-9}		E2
Watson et al. 2005 [72]	Rat	GH3/B6/F10 GH3/B6/D9	Intracellular Ca^{2+}, Prolactin release	Ringer's solution, Culture medium	1, 3 min	10^{-12}	10^{-12}, 10^{-10}*, 10^{-9}*, 10^{-8}*, 10^{-7}		Coumesterol; DDE; DES; dieldrin; endosulfan; *p*-NP
Wozniak et al. 2005 [77]	Rat	GH3/B6/F10 GH3/B6/D9	Intracellular Ca^{2+}, Prolactin release	Ringer's solution, Culture medium	0–20 min, 1–15 min	10^{-12}*	10^{-12}*, 10^{-10}*, 10^{-9}*, 10^{-8}*, 10^{-12}*, 10^{-11}*, 10^{-10}, 10^{-9}, 10^{-8}*	Nifedipine	Coumesterol; DDE; DES; dieldrin; endosulfan; *p*-NP

complex, and depend on the recruitment of tissue-specific co-regulatory factors that differentially affect the interaction of ERs with EREs of different target genes [91,92]. BPA selectively binds to ERα and ERβ, and has a higher affinity for ERβ in target cells [6–8]. Kuiper et al. reported a Ki for BPA of 195 and 35 nM at ERα and ERβ, respectively [6]. It was also found that the binding affinity relative to 17β-estradiol for BPA at ERβ was 6.6-fold higher than at ERα; 0.33 and 0.05, respectively [6]. Satoh et al. also showed that BPA could bind to human ERα and reported an IC_{50} (the concentration of chemical required to reduce specific 17β-estradiol binding by 50%) of 7.8 μM [12]. Gould et al. demonstrated that as an endocrine disruptor BPA is not merely a weak estrogen mimic, but it exhibits characteristics of a distinct molecular mechanism of action at ERα, with BPA interacting differently within the ligand-binding domain [5]. These findings reveal that the interactions of BPA and 17β-estradiol with an estrogen receptor can induce differential molecular effects, thus ultimately impacting the physiological response of sensitive cells.

In addition to the differential ER binding properties, there are major differences in the effects of BPA on co-activator recruitment by ERα and ERβ [8]. Specifically, the BPA/ERβ receptor/ligand complex showed over 500-fold greater potency than the BPA/ERα complex in recruiting the co-activator TIF2. Moreover, the enhanced (relative to ERα) ability of ERβ to recruit co-activators in the presence of BPA was consistent with a greater capacity of ERβ to potentiate reporter gene activity in transiently transfected HeLa cells expressing ERβ and TIF2 over a concentration range of 1–100 μM [8]. A higher relative binding affinity (RBA) for BPA at ERβ was also observed (estradiol RBA for BPA at ERβ was 0.75 versus 0.073 for ERα). Based on those data it was concluded that ligand-dependent differences in the ability of ERα and ERβ to recruit co-activator proteins would contribute to the complex tissue-dependent agonistic or antagonistic responses observed with exposure to endocrine disruptors such as BPA.

Numerous isoforms of ERα and ERβ have been identified and they likely play unique roles as mediators of the actions of endogenous estrogens and BPA. For example, Ramsey et al. used different ERE-sequence containing reporter-constructs to demonstrate that expression of different isoforms of ERβ differentially impacted ERE-regulated gene expression in a ligand dependent manner [93]. These results suggest that the cell-type specific expression of different ER isoforms together with availability of a specific ligand, contributes to the cellular complexity of ERE-regulated estrogen responsive target gene expression. Moreover, a study by Masuyama et al. showed that compared to 17β-estradiol, BPA blocks the ubiquitination and degradation of ERβ, suggesting that BPA may affect ERβ-mediated transcription of target genes by differential degradation of nuclear receptors [94].

8. Differential responsiveness of sensitive cells and tissues

Regarding cell-specific differences in response to BPA, it was proposed that the nature of the estrogen-responsive promoter would also influence the activity of endocrine disrupting chemicals through ERs, similar to the way in which SERMs manifest their gene-specific activities. The idea that ligands play an active role in ER function was proposed by McDonnell and others who demonstrated that different ligands induce distinct conformational changes in the ER, correlating with the observed diversity in transcriptional and biological responses [95–97].

Pennie et al. showed that the nature of the ERE, along with the ratio of ERα and ERβ in a particular cell or tissue could influence ER-responsive genes in the presence of estrogenic compounds such as BPA [14]. In those studies, the effects of BPA at doses from 1 to 100 μM and 17β-estradiol (1 nM to 1 μM) in COS-1 cells transiently transfected with either vitellogenin ERE or LHβ ERE reporters were characterized. A follow-up study by Hall et al. confirmed these findings by demonstrating that the transcriptional activities of EDCs are mediated in a promoter-specific manner, and that estrogen-responsive promoters communicate differentially with ERα and ERβ by influencing the receptor structures, thus resulting in a diversity of transcriptional responses [13]. Specifically, HepG2 (human hepatoma), Ishikawa (human endometrial carcinoma) or UCI107 (human ovarian carcinoma) cells were co-transfected with ERα or ERβ expression plasmids together with different firefly luciferase reporter constructs. The reporter constructs contained either three copies of the vitellogenin ERE, the estrogen-responsive promoters from pS2, lactoferrin, or complement 3 (C3). Notably, BPA action through ERβ was significantly influenced by the nature of the promoter utilized. In particular, BPA displayed remarkably high potency on pS2 promoter through ERβ, with higher ERα-mediated activity at vitellogenin and lactoferrin promoters [13]. The authors concluded that different estrogen-responsive promoters may influence the ability of the ER to form a transcriptionally active conformation, providing a mechanism for the promoter-specific activities observed. These studies are in agreement with Kurosawa et al. who demonstrated that BPA exhibits estradiol-like agonist activity via ERβ, and has mixed agonist and antagonist activity in some types of cells via ERα [16]. Thus, for a given cell type, the activity of BPA likely depends in part on the levels of different ER isoforms and variants expressed in the sensitive cell or tissue.

Although altered ER-regulated gene expression through the ERE is a major mechanism of BPA action, recently it was shown by Singleton et al., that high concentrations of BPA could regulate expression of a distinct set of genes involved in growth and development, such as HOXC1 and C6, Wnt5A, Frizzled, TGFβ-2, and STAT inhibitor 2 in MCF-7 breast cancer cells stably expressing HA-tagged ERα [98]. When HOXC6 mRNA expression was examined by quantitative RT-PCR, three independent ERα-HA cell cultures showed down-regulation (2.2–2.8-fold) following 3 h treatment with 1 μM BPA. HOX genes encode transcription factors containing the highly conserved 183-base-pair homeodomain sequence and are critically important for embryonic development [99]. Although biological functions of the HOXC6 gene product are not well defined, targeted disruption of this gene in mice has indicated a role in mammary ductal development [100]. In contrast to Singeleton's study, Buterin et al. demonstrated that exposure of MCF-7 and T47D breast

cancer cells to pharmacological concentrations of BPA (10 μM) resulted in identical transcriptional effects as induced by physiological concentrations of estradiol [101]. These authors suggest that endocrine disrupting compounds may act in conjunction with endogenous steroid hormones to induce additive effects in target tissues. Another study demonstrated that exposure to low BPA doses (ranging from 0.1 nM to 25 μM) increased HOXA10 protein expression in the uterus [102]. Thus, the results from these studies reveal novel non-ERE-regulated effects on gene expression by BPA, suggesting a role for additional transcriptional mechanisms of BPA action in reproductive and mammary gland development.

In order to identify tissues and cells that contain functionally active ERs, and to define their ability to respond to different ligands, Nagel et al. developed ER action indicator (ERIN) transgenic mice (strain C57BL/6-SJL). This model system integrates the upstream requirements of ER action, including the receptor, ligand, and accessory comodulators with activation of the ER, resulting in expression of the enzyme β-galactosidase (β-gal), which allows for enzymatic amplification of the estrogenic signal and histological localization of its activity [103]. In these studies, BPA elicited a distinct cellular profile of gene expression, similar to SERMs. BPA stimulated ER transcriptional activity at a dose of 800 μg/kg (40% efficacy as compared to DES-maximum) and exhibited 20% of DES-induced maximum response at a low dose of 25 μg/kg; however, BPA had little uterotropic activity in this system [103]. In contrast, in the same model, the well-established SERM tamoxifen significantly increased uterine weight at 25 mg/kg, while minimally inducing ER reporter activity in this tissue [103]. The authors suggest that these differential actions of BPA may be due to this ligand's selective ER modulatory activities, resulting in tissue-selective ER activity.

In agreement with Nagel and colleagues, a recent study by Hong et al., also demonstrated distinct expression patterns of responsive genes following exposure to either estradiol or endocrine disrupting compounds, including BPA, thus further implicating differential effects of estradiol versus BPA in reproductive tissues [104]. Based on the studies described above, it is likely that a BPA-induced ER conformation results in recruitment of cell- and tissue-specific co-modulatory factors responsible for the differential responses to this ligand.

9. BPA effects on cells and tissues of the reproductive system

The findings described above raise further questions about the differential roles of endocrine disruptors in human development and reproduction. Based on recent molecular studies, it was proposed by Welshons et al., that BPA is a SERM [4]. An investigation into SERMs/endocrine disruptors confirmed that BPA effects in uterus, vagina and the bone are not purely estrogenic. Utilizing low doses of BPA (37 and also 370 μg/kg/body weight) in Sprague–Dawley rats, Seidlova-Wuttke et al. found no significant effect on uterine weight, but a clear increase in epithelial thickness, which was contrasted by a reduction in the myometrial thickness [11]. Following 3 months treatment with 37 μg/kg body weight/day BPA, a modest reduction in endometrial thickness was observed. These effects were at much lower doses than used in previous experiments [105]. Hence, the uterotrophic assay in this study revealed subtle, low-dose effects of BPA that cannot be defined as "classical" estrogenic effects. Seidlova-Wuttke et al. also used quantitative RT-PCR analysis to characterize the effects of BPA on ERα, ERβ and C3 transcripts in the uterus. It was found that estradiol had no effect on ERα mRNA, decreased ERβ mRNA, and greatly increased C3 mRNA expression. Interestingly, these effects were not mimicked by BPA. In fact, BPA exposure increased ERα, did not effect ERβ, and decreased C3 mRNA expression [11]. Treatment with both 37 and 370 μg/kg BPA also resulted in significant reductions of bone mineral density, which was in stark contrast to estradiol, whose action is essential for maintenance of normal bone structure. The authors suggest that BPA-induced effects on bone metabolism were through mechanisms other than those regulated by ERα or ERβ. It is known that the bone expresses the aryl hydrocarbon receptor (AhR) [106,107], but whether BPA serves as a ligand for the AhR still needs to be established. While the rapid signaling effects of BPA on bone cells have not been investigated, sex-specific rapid effects of estradiol on intracellular signal transduction in osteoblasts are well documented [108], and the signaling network for the rapid actions of the phytoestrogen diadzein has also been described [109]. Thus, it is likely that BPA acts via membrane ER isoforms, contributing to regulation of bone physiology. Additionally, BPA may modulate the available co-regulatory factors (enhancers or repressors of ER) in the uterus and bone. Clearly, further investigations are needed to decipher BPA's molecular mode of action in these tissues.

Based on studies described in this section, it is evident that some actions of BPA are compatible with its characterization as a SERM. However, mounting research has demonstrated that BPA cannot be defined exclusively as a SERM. The actions of BPA extend beyond its ability to mimic, enhance, or inhibit the activity of endogenous estrogens at the estrogen receptor: as described below, BPA is not ER "selective," because it also binds and affects other nuclear receptors, including the androgen and thyroid receptors.

10. BPA effects related to fertility

Numerous studies have implicated BPA and other EDCs in reduced female fertility and the role of estrogens in oocyte development is a topic of considerable research interest [110]. It is known that theca cells of the late-stage follicle express both the ERα and ERβ, whereas the granulosa cells express predominantly ERβ. Accordingly, BPA has the potential to affect both theca and granulosa cells, however the degree to which the differential expression of ERα and ERβ, and the differences in receptor affinity for BPA might impact these ovarian cells is unknown [6–8,11].

In studies using a primary culture system of human endometrial cells, inhibition of cell growth was observed following exposure to 1 μM BPA [25]. Granulosa cells express ERs and are essential for ovarian follicullogenesis and steroidogenesis (reviewed in [111]). The impact of BPA, at low 0.1 nM dose,

on murine ovarian granulosa cells was investigated by Xu et al., who found that this low and physiologically relevant dose of BPA decreased granulosa cell viability by inducing apoptosis and G2-to-M cell cycle arrest, suggesting that granulosa cells are highly sensitive to BPA [112].

In contrast, others have reported that pharmacological concentrations of 17β-estradiol (1 μg/mL) exert antiapoptotic effect on ovarian granulosa cells [113,114]. Based on previous findings demonstrating that BPA could partially antagonize the effects of 17β-estradiol at ERα, but not ERβ [115], Xu et al. (2002) suggested that their results can be explained by BPA's ability to antagonize antiapoptotic effect of intrinsic estrogens produced by granulosa cells. While these researchers showed that BPA increased the expression of Bax and concomitantly decreased the expression of Bcl2 at both protein and mRNA levels, the precise mechanism of BPA action to induce apoptosis in granulosa cells still remains to be determined.

An *in vivo* study by Hunt et al., found that chronic low-dose exposure to BPA causes meiotic aneuploidy in mouse oocytes, thus implicating BPA in disruption of female meiosis. Specifically, this study demonstrated that low oral BPA exposure to BPA at 20, 40, or 100 μg/kg body weight/day for 6–8 days preceding the analysis, adversely affects oocyte chromosome segregation [116]. The molecular mechanism(s) through which BPA may exert effects on meiosis is an important research topic that is in need of further investigation.

11. BPA actions at the androgen receptor

Several studies using *in vitro* yeast-based assays revealed that BPA exhibits strong anti-androgenic activity [23,29]. Using ligand competition assays, it was demonstrated that BPA could compete with 5α-dihydrotestosterone (DHT) for binding at the androgen receptor (AR) with an IC_{50} (the concentration of chemical required to reduce the specific DHT binding by 50%) value of 2.14 μM [24,29]. By utilizing an elegant yeast two-hybrid system, Lee et al. demonstrated that BPA affects AR interaction with its co-regulator ASC1, to subsequently modulate AR-transactivation. In addition, analysis of GFP-AR fusion protein in the presence of BPA showed inhibition of AR nuclear localization [29]. These findings suggest that BPA can affect multiple steps of the activation and function of AR, including modulation of AR interaction with its co-regulator ASC1 and inhibition of the binding of native androgens to the AR, resulting in antagonistic action of BPA on the AR.

A recent study by Xu et al. has confirmed that BPA can act as an anti-androgen in a mammalian system [27]. In this study, a human androgen receptor (hAR) reporter gene assay using African monkey kidney cell line CV-1 transiently transfected with the reporter gene plasmid pMMTV-CAT was utilized. BPA showed significant inhibitory effects on the transcriptional activity induced by DHT with an IC_{50} value of ~0.8 μM; the highest anti-androgenic activity of any endocrine-disrupting compounds tested [27].

Using two stably transfected Chinese hamster ovarian cell lines (CHO-K1 cells) as an AR-EcoScreen for androgenic activity and c-luc for cell toxicity evaluation, Satoh et al. investigated *in vitro* hormonal activity of BPA and its derivates bisphenol A diglycidyl ether (BADGE) and bisphenol F diglycidyl ether (BFDGE) [12]. These BPA derivatives are used as interior coating for food cans and components of some dental sealants. One of the CHO-K1 derived cell lines stably expressed androgen-inducible luciferase, while the other stably expressed luciferase independent of androgen induction. It was found that BPA, BADGE and BFDGE exhibited anti-androgenic activity, suggesting that chlorohydroxy compounds such as BADGE and BFDGE act as androgen antagonist through binding to the AR. BPA had a potent binding affinity for AR, and inhibited specific testosterone binding to the receptor in a concentration-dependent manner. The IC_{50} value for AR was 7.9 μM, and complete inhibition appeared at 19 μM [12]. A follow-up study by Roy et al., using CHO-K1 stably expressing hAR and a mouse mammary tumor virus-neomycin-luciferase reporter gene, confirmed that BPA is an anti-androgenic compound with an IC_{50} = 19.6 μM [22]. However, a study by Gaido et al., failed to demonstrate antiandrogenic activities of BPA in HepG2 cells [117].

Thus, research results show that BPA can act as AR antagonist in some cell systems. Once bound to AR, the AR/BPA complex may prevent endogenous androgens from regulating androgen-dependent transcription. The specificity of AR–ligand interaction may be critical in eliciting adverse effects on male reproductive system. However, discrepancies between different research groups on antiandrogenic activities of BPA exist – this area of research requires, further investigation.

Similar to endogenous estrogens, several endocrine disrupting compounds have been hypothesized to alter androgen action or prostate growth and development [118]. In fact, developmental (*in utero* or neonatal) exposure to low dose BPA and other compounds, including polychlorinated bisphenols (PCBs), hexachlorobenzene (HCB) and diethylstilbestrol (DES), has been reported to increase prostate size and weight, suggesting that these agents influence early prostate growth patterns [119–125]. Although the mechanism of BPA action in the prostate is yet to be determined, recent studies have shown its ability to influence AR function and activity.

Given the importance of the AR for prostate development and tumorigenesis, several studies monitored effects of BPA exposure on AR expression and function *in vivo*. While two studies demonstrated that both BPA (25 or 250 μg/kg/day) and estradiol (0.02–2 μg/kg) increased AR expression in dorsolateral and ventral prostate lobes [123,126], a report by Ramos et al. observed no change in the ventral prostate upon BPA exposure [122]. However, the differentiation pattern of periductal stromal cells of the ventral prostate was found altered by prenatal exposure to BPA at 25 or 250 μg/kg/day [122]. Together, the mitogenic effect of BPA on prostate cells often correlates with changes in the AR status, although future studies are needed to elucidate the relationship between BPA and AR in the developing prostate.

The impact of BPA on AR regulation in prostate cancer cells and the response to androgen-deprivation therapy (ADT) has also been examined. Because of the androgen dependence of prostatic adenocarcinomas, the mainline therapeutic intervention in advanced prostate cancer is ADT. Although ADT

is initially highly effective, within 2–3 years of therapy most patients develop ADT-resistant tumors, for which no effective treatment is available [127,128].

It was shown by Wetherill and colleagues that low concentrations of BPA (1–10 nM) are capable of activating the mutant AR (AR-T877A), that is selected for during ADT therapy in prostate cancer cells. Under conditions of androgen ablation, BPA activation of the mutant AR induced cellular proliferation [26]. In contrast, BPA has no agonistic activities on the wild-type AR [23,24,29]. Wetherill et al. also demonstrated that BPA competes with DHT for AR-T877A binding by a non-competitive mechanism, and enhances mutant AR activity in both androgen-dependent (mutant AR-T877A) and androgen-independent (mutant AR-H874Y) prostate cancer cell lines in the presence of DHT [28]. In contrast, in an androgen-dependent prostate cancer cell line LAPC-4 that expresses a wild-type AR, low doses of BPA only marginally activate the receptor and fail to show additive effects with DHT on AR transactivation [28]. In addition, this group showed that the ability of BPA to activate tumor-derived AR is conserved across multiple mutant ARs (AR-V715M, AR-T877S, and AR-H874Y), as demonstrated by both yeast-based and mammalian cell assays. These mutations in the AR were identified in patients with metastatic androgen-independent prostate tumors following ADT [129,130]. At higher doses of 1–10 μM BPA, growth inhibition of androgen-dependent prostate cancer cell lines (LNCaP and LAPC-4) was observed. Thus, the differential effects of lower and higher doses of BPA were demonstrated, as the growth inhibitory effect at higher doses is seen only in androgen-dependent prostate cancer cells [28]. These results are in agreement with previous studies that showed inhibition of AR activity at high dose exposure to other endocrine disruptors [131,132] and BPA [22–24,27,29,35]. Collectively, the above *in vitro* studies, offer a mechanism by which environmentally relevant BPA exposures in prostate cancer might lead to tumor cell proliferation and suggest that BPA can interfere with therapy in prostate cancer patients by activating mutant ARs that are frequently selected for during ADT; these effects of BPA could contribute to the disease relapse. In fact, a recent study by Wetherill and Hess-Wilson et al. suggests that exposure of advanced prostate tumors expressing specific somatic mutations (AR-*T877A*) in the androgen receptor gene to environmentally relevant doses of BPA may facilitate ADT bypass and advance tumor recurrence [133].

12. Effects of BPA on cells and tissues of the male reproductive system

A study in adult Leydig cells by Akingbemi et al. demonstrated that low-dose BPA (0.01 nM) decreases testosterone biosynthesis by 25% as a result of decreased expression of the steroidogenic enzyme 17α-monooxygenase (17alpha-hydroxylase/17–20 lyase) [134]. Further, post-natal exposure to BPA decreased serum 17β-estradiol levels in Long-Evans rats exposed to 2.4, 10, or 100 μg/kg/day of BPA by inhibiting Leydig cell aromatase activity and exposure to lowest and environmentally relevant BPA dose (2.4 μg/kg/day) decreased the mRNA levels of lutenizing hormone (LH)-β and increased ERβ expression in the pituitary [134]. These findings demonstrate that the perinatal period is a sensitive window of exposure to BPA in this animal system. The effects of BPA on steriodogenesis in Leydig cells was also analyzed by measuring the LH receptor-mediated cAMP and progesterone production in cultured mouse Leydig tumor cells [135]. This study found that BPA inhibited cAMP production and progesterone synthesis by preventing coupling between LH receptor and adenylate cyclase. However, estradiol did not inhibit cAMP production, thus further emphasizing the complexity of endocrine disruption.

In the testis, Sertoli cells establish intercellular junctions that are essential for spermatogenesis. The SerW3 Sertoli cell line displays some features of native Sertoli cells. Fiorini et al. studied the mechanism of BPA action on Sertoli cells [136]. Using Western blot and immunofluorescence analyses it was found that SerW3 Sertoli cells express typical components of tight (occludin and zonula occludens-1), anchoring (N-cadherin) and gap (connexin 43) junctions. Similar to other endocrine disruptors, BPA affected intercellular junctions by either reducing their amount, or by inducing aberrant intracellular localization of these membrane proteins [136].

Song et al. investigated the action of BPA on orphan nuclear receptor Nur77 gene expression and steroidogenesis, as it plays an important role in the regulation of LH-mediated steroidogenesis in testicular mouse K28 cells [137]. The K28 mouse Leydig tumor cell line has been characterized as a suitable cell culture model for steroidogenesis [138,139]. It was found that exposure to 10 nM BPA induces Nur77 gene expression in K28 cells after 30 min treatment and BPA-mediated induction of Nur77 gene expression is ERK mitogen-activated protein kinase (MAPK) and PKA dependent. At high BPA concentrations (1 μM), progesterone biosynthesis was increased in K28 cells; this effect was suppressed by overexpression of dominant negative Nur77. Moreover, high dose BPA effects on steroidogenesis were confirmed *in vivo* where BPA injection into prepubertal mice (25 mg/kg; 18-day old) resulted in induction of Nur77 mRNA and increased concentrations of testicular testosterone *in vivo* [137]. Taken together, these results demonstrate that BPA can induce Nur77 gene expression, which subsequently alters the steroidogenesis in testicular Leydig cells. Post-natal exposure to a low dose of BPA (2.4 μg/kg/day) was also found to reduce expression of cortactin, an actin-binding protein, in mouse testes [140]. Thus, the studies described above implicate adverse effects of low dose environmental BPA exposure on male fertility.

13. Thyroid hormone action, thyroid receptor and BPA

Thyroid hormone (TH) is essential for normal brain development, and recent studies indicate that the developing brain is very sensitive to small perturbations in thyroid hormone status. As a result, understanding the action of environmental chemicals that can interfere with TH signaling mechanisms is highly important to human health. The first environmental chemical shown to bind to the thyroid hormone receptor (TR) was BPA and its brominated and chlorinated derivatives. It was demonstrated by Meerts et al. in 2001 that polybrominated BPA compounds have estro-

genic activities and are able to bind to the ERα [141]. A study in 2002 by Kitamura et al. showed that BPA, tetrabromo- and tetrachloro-BPA (TBBPA ad TCBPA, respectively) could displace thyroid hormone ^{125}I-T_3 from cell nuclei isolated from the estrogen-responsive pituitary cell line MTt/E-2 [33]. Although these data indicate TR binding, it is not clear whether this binding is specific to the TRβ, TRα or both. In the thyroid hormone-responsive pituitary cell line GH3 (in which the growth hormone secretion depends markedly on thyroid hormones, but little on estrogen), TCBPA and TBBPA increase cell proliferation at the dose of 100 μM and 1–100 μM, respectively [33]. These results indicate that brominated BPA derivatives act as TR agonists in pituitary GH3 cells. Further support for this interpretation was the observation that these two halogenated BPA compounds increase GH3 cell proliferation, and produce growth hormone (GH) production in the presence of thyroid hormone T3 in an additive way. In contrast, Moriyama et al., who utilized rat liver nuclei that express both the TRα and TRβ, showed that BPA binds to the TR and acts as an antagonist on this receptor [36]. This conclusion was based on two types of experiments. In the first approach, they fused the ligand binding domain (LBD) of either TRα or TRβ to the Gal4 DNA binding domain and performed *in vitro* expression assays using a Gal4 response element. It was found that BPA at 1.0 μM inhibited both the TRα- and TRβ-stimulated response induced by thyroid hormone T_3. In the second approach, investigators co-transfected full length TRα and TRβ into TSA-201 cell (a clone of human embryonic kidney 293 cells) with one of three types of thyroid hormone response elements (TRE): a palindrome, the malic enzyme TRE, and the TSHα TRE. They found that in the presence of both TRα and TRβ, BPA produced a dose-dependent inhibition in the T_3 response (10 nM T_3) on both the palindromic and the malic enzyme TRE, but produced a dose-dependent induction in the T_3 response on the TSHα TRE. Because the TSHα TRE is a negative response element, the authors speculated that BPA stabilized the interaction between TRα1 or TRβ1 and a nuclear repressor N-CoR. To test this hypothesis, Moriyama et al. performed a mammalian two-hybrid experiment using GAL4-NCoR and TR-VP16 with their GAL4 reporter construct in TSA-201 cells. These experiments fully supported the concept that BPA binding to TR stabilized the interaction between the TR and N-CoR. This hypothesis that is consistent with the observations that BPA suppresses T_3 action on positive TREs (malic enzyme and a DR4), and enhances T_3 action on a negative TSHα TRE.

The above studies indicate that BPA is an antagonist on the TR, but that halogenated BPA derivatives (commonly used as flame retardants) are TR agonists. Moreover, the affinity of the TR for brominated TBBPA or TCBPA appears to be higher than for BPA. Finally, these data indicate that BPA and its halogenated derivatives can bind two classes of TRs equally well. However, the latter concept is inconsistent with the work of Ghisari and Bonefeld-Jorgensen where BPA and TB-BPA were found to exert thyroid hormone-like activity in GH3 cells by increasing cell proliferation [30]. Interestingly, BPA, but not TBBPA, increased GH3 cell proliferation in the presence of a pure estrogen receptor antagonist ICI 182,780, suggesting that ER-mediated transactivation is not involved and that BPA may exert effects directly on the TR [30]. Other studies have found that TBBPA has a lower affinity for the ER than does BPA [142]. It is unclear why BPA appeared to have a stronger action on the TR than TB-BPA in this study. Moreover, BPA appeared to act as an agonist, which is not consistent with previous *in vitro* studies.

The contribution of rapid intracellular signaling effects are well described for T_3, estradiol, and BPA and cannot be ruled out as contributing to the actions of BPA in this cell model system (reviewed in [143,144]). A potential contribution of rapid signaling effects in TH/TR-mediated effects of BPA and/or TB-BPA are supported by the work of Canesi et al. who showed that BPA (25 μM) and TBBPA (5 μM) stimulate rapid and transient activation of ERK-MAPK and PKC in hemocytes isolated from the marine mussel *Mytilus galloprovincialis* [75]. While these studies reinforce the potential contribution of rapid signaling to BPA-mediated actions at the TRs, the high concentrations of BPA required to activate signaling effects limit their significance.

Other halogenated BPA derivatives were shown to act as TR antagonists in the study by Kitamura et al. [34]. The investigators used CHO cells in a transient transfection assay in which human TRα or TRβ were co-transfected with a palindromic TRE. In these experiments, TBBPA and TCBPA were potent antagonists of T_3, producing significant inhibition of T_3 action at concentrations as low as 3 μM, but this action depended upon the compound and the TR isoform. In general, TBBPA was more potent than TCBPA, and TRα was more sensitive to each of these compounds. These findings are not consistent with the initial studies reviewed above in which the halogenated BPA derivatives acted as agonists and parent BPA acted as an antagonist. Differences in the cellular context (i.e., use of different cell lines), and/or differences in the reporter construct (i.e., the use of a DR4 versus a palindrome) may account for the reported experimental differences.

Kitamura et al. followed these observations with a comparison of effects of BPA, TBBPA and TC-BPA on ER, AR and TR signaling [35]. This study supported their previous observations that halogenated derivatives of BPA act as TR agonists in pituitary GH3 cells [35]. Interestingly, the biologically active dose in these experiments was one or two orders of magnitude higher than doses producing estrogenic or androgenic effects, concentrations ranged from 1 to 100 μM. In contrast to previous studies by Moriyama et al. [36], an agonistic or antagonistic action of BPA on the TR in this system was not observed.

Hamers et al. have profiled a number of polyhalogenated aryl hydrocarbons for their ability to signal through the AhR receptor, ER, AR, and TR [31]. In addition, they characterized the ability of these compounds to displace T_4 from transthyretin, or to affect estrogen sulfotransferases. In these studies, TB-BPA was a very potent T_4 competitor in the TTR-binding assay ($IC_{50} < 0.1$ μM) with a 1.6 times higher TTR-binding potency than the natural ligand T_4. In their GH3 cell-based assays, BPA, TB-BPA and TC-BPA were nearly inactive, yet TB-BPA was found to potentiate the effects of T_3 in the T-screen assay.

Finally, Schricks et al. modified the T-screen to produce a more quantifiable assay [145]. This assay utilized a GH3 rat pituitary cell line that specifically proliferates when exposed to thyroid hormone T_3. The growth stimulatory effect is mediated

via T_3-receptors. In this proliferation assay, resozurine is used to measure cell proliferation. It was found that neither BPA nor the halogenated derivatives were active as agonists or antagonists in this system. However, it was shown that BPA significantly potentiated the effect of T_3.

Taken together, *in vitro* studies demonstrate that BPA binds to both the TRα and TRβ with relatively low affinity. In addition, the halogenated flame retardants, TB-BPA and TC-BPA bind with higher affinity. However, some studies indicate that BPA acts as an antagonist on the TR [32,34–36], whereas other studies conclude that BPA acts as an agonist [30,33], and some studies indicated that BPA and its halogenated derivatives are not active on thyroid hormone signaling. It is unclear why such differences in experimental results were obtained. Several investigators have employed GH3 cells in their studies, and have still obtained different results. This could be partially due to experimental design differences: initial reports utilized the MTT assay to evaluate relative growth, as well as growth hormone production (GH is controlled by thyroid hormone) [33], whereas in later studies the authors used a modified version of a cell proliferation assay (the use of resazurine), which may differ in its sensitivity [145]. It is also important to consider the possibility that differences in cell lines may have developed and that the differing results might reflect differences in transcriptional machinery that impact the ability of the TR to drive gene expression. If these compounds bind to the TR but produce a slightly different conformational response in the protein, it is theoretically possible that the TR will interact differentially with various cofactors. The initial observation of Moriyama et al. indicating that BPA can alter the strength of the interaction between TR and NCoR supports this interpretation [36].

If BPA and its halogenated derivatives interfere with thyroid hormone signaling, *in vivo* effects that are consistent with this concept might be observed. In this regard, Iwamuro et al. found that BPA reduces the rate of metamorphosis in *Xenopus* and inhibites T_4-accelerated metamorphosis [146]. These investigators also showed *in vitro* that T_4-induced tail resorption inhibited by BPA in a dose-dependent manner. These findings are fully consistent with the hypothesis that BPA acts as an antagonist of the *Xenopus* thyroid receptorthyroid receptor. This finding is consistent with those of Kitamura et al. where T_3-induced tail shortening is inhibited by TB-BPA in a Ranid [34]. Further, Kudo et al. found that 3,3′,5-tribromobisphenol A and TB-BPA both exhibited T_3 agonist and antagonist activities at submicromolar concentrations in a *Xenopus laevis* cell line and in tadpoles [147]. These compounds competed with T_3 binding to xTTR and xTR *in vitro*, suggesting these proteins are possible target sites for BPA action *in vivo*. In addition, the dose at which TR signaling was influenced was lower than the IC_{50} values for the receptor. Therefore, the authors suggest that novel molecular mechanisms of interaction with the TR may be at play. Interestingly, Sugiyama et al. reported that TCBPA could inhibit the effect of T_3 on a luciferase construct driven by a *Xenopus* TRE (the TH/bZIP TRE), but that TB-BPA acts as a TR agonist in the absence of T_3 [148].

Iwamuro et al. investigated the effects of BPA on the expression of TR-α and TR-β, and retinoid X receptor (RXR) gamma mRNA in tails of stage 52–54 *Xenopus* tadpoles in organ culture in the presence or absence of different concentrations of T_3 [32]. In the absence of T_3, BPA at all concentrations examined did not show remarkable effects on tail length, but blocked T_3-induced tail resorption in a concentration-dependent manner. Changes in expression of TRα and TRβ mRNAs from the tail specimens (measured by RT-PCR) indicated that low BPA dose (0.1 μM) showed an apparent antagonistic effect towards the receptors and reduced mRNA levels. When administered together with T_3, the antagonistic effects of BPA were more pronounced. Autoinduction of both TRα and TRβ genes by T_3 was inhibited by BPA, but the effect was less marked on TRα than on TRβ. Gene expression of RXRγ, a partner for heterodimer formation of TRs, was moderately suppressed either by T_3 or by BPA, but no additive effects were observed.

Those studies show that somewhat different findings are reported using amphibian models as well as those reviewed above for other *in vitro* models. Although some of these data appear conflicting, it is clear that BPA and its halogenated derivatives can interfere with TH signaling in the amphibian model. The differences among studies characterizing these compounds as agonists or antagonists likely reflect the complexity of mechanisms underlying BPA actions on the TR.

Findings from studies in mammals are also complex. For example, Zoeller et al. [37] reported that BPA could increase serum total T_4 in rat pups, but also increased RC3 expression in the dentate gyrus. Their interpretation was that BPA acted as a selective TRβ antagonist, causing an increase in serum T_4. However, because RC3 expression is driven by the TRα in the dentate gyrus, these authors speculated that BPA was not exerting the same action on the TRα. In contrast, Nakamura et al., who studied whether prenatal exposure to low-doses of BPA by injection of pregnant mice with 20 μg/kg/day BPA affected the brain morphology and expression of genes related to brain development, reported that BPA exposure causes changes in the histogenesis of the cerebral cortex and increases the expression of a number of genes, including the TRα gene. These results were interpreted as reflecting a thyroid hormone-like effect of BPA in the cortex [149]. A similar observation was also made in *Xenopus* where TB-BPA increased TRα, but not TRβ, expression [150].

These studies suggest that BPA and a variety of its derivatives interfere with thyroid hormone signaling during development. The mechanism(s) underlying these effects are likely to be complex given that the dose of BPA required to produce effects on molecular events may be lower than the IC_{50} for binding to the thyroid receptor. It is also likely that BPA and its derivatives alter the relationship of the TR to various co-modulatory molecules. Moreover, BPA and its derivatives may also influence the abundance of the receptor. Thus, the mechanisms associated with BPA endocrine disrupting effects on the TH system are currently ill-defined and warrant further detailed investigation.

14. BPA and rapid signaling systems

BPA also exerts cell- and tissue-specific effects that act through mechanisms which modulate a variety of cell signaling pathways. The impact of BPA exposure is observed within

seconds to minutes, and is initially independent of the nuclear hormone receptor mediated transactivation activity that induces later effects on gene expression. However, these rapid signaling pathways interact with the traditional nuclear hormone receptor pathways [144,151].

For specific cell types, the features of the mechanisms associated with rapid signaling for estradiol, thyroid hormone and some endocrine disruptors, including BPA, are becoming more understood with recent research findings showing the presence of extracellularly accessible binding sites that act to modulate intracellular signaling [12,143,144,152]. Although the properties of the receptors involved in the initiation of rapid signaling effects in different tissues/cells are often related to those of a nuclear hormone receptor-like protein [20], some receptor/signaling pathways can be explained by an alternative transmembrane heptahelical G-protein coupled receptor [153]. A candidate G-protein coupled membrane estrogen receptor (GPR30) that binds estradiol and the endocrine disruptor *ortho,para*-dichlorodiphenyldichloroethylene (*o,p'*-DDE) with relatively low affinities has been described [71]. At GPR30, an IC_{50} for BPA was found to be 630 nM and its RBA (when compared to 17β-estradiol) equals 2.83 nM [79]. It is notable that the RBA for BPA at GPR30 is higher than that observed for ERα or ERβ. In addition, this study demonstrated that following a 30 min treatment, both estradiol and 200 nM BPA increase cAMP activity, a GPR30-dependent signaling pathway activated by estrogens, in stably transfected ER-negative HEK 293 cells [79]. Thus, it is likely that BPA exerts some effects through this novel seven-transmembrane estrogen receptor GPR30, although the consequences of inappropriate activation of this signaling pathway are currently unknown. It is important to caution that even in controlled recombinant over-expression systems, the role of GPR30 receptor in rapid estradiol-mediated signaling is still controversial [154,155].

14.1. Rapid signaling effects in pituitary cells

Investigations of potential impact of BPA on pituitary physiology have been largely focused on examining pituitary tumor cell growth and disruption of prolactin release. Established GH3 rat pituitary tumor cell line has been a valuable model in characterizing the mechanisms involved in the estrogenic release of prolactin [156]. Steinmetz et al. demonstrated that 1 μM BPA stimulates prolactin release in GH3 cells and induces hyperprolactinemia in F344 rats [157]. Watson et al. identified an extracellularly accessible plasma membrane ER on GH3/B6 rat pituitary tumor cell line [158,159], and have concluded that concentrations of BPA as low as 1 fM can cause an influx of calcium from extracellular sources via nifedapine-sensitive L-type calcium channels within 1 min of exposure [20,72,77]. In addition, these low concentrations of BPA could induce rapid release of prolactin from pituitary cells; effects that are mediated by the elevation of intracellular calcium and similar to those observed for estradiol [77]. Because immunoselected or limiting dilution selected GH3/B6 cells with very low expression levels of a cell surface ERα epitope were non-responsive, these rapid signaling effects of BPA were associated with expression of an extracellular accessible ERα-like protein. In this GH3/B6 cell model, several other potential endocrine disrupting compounds (DDE, coumestrol, endosulfan, and nonylphenol) induced small increases in ERK1/2 phosphorylation; however, rapid increases in ERK-phosphorylation were not observable in response to 1 nM BPA [68,69].

In contrast to the results from the GH3 cell model, rapid modulation of ERK-signaling in response to BPA has been observed in neurons and immune cells; observations lending further support for the cell/tissue specific effects of BPA. For example, Canesi et al. showed that concentrations of BPA on *Mytilus* hemocytes induced small, but significant, and transient increases in ERK1/2 and PKC pathways with later increases in ERK2 and STAT3 phosphorylation [75]. There results are in agreement with a study of tetrabromobisphenol A (TB-BPA) toxicity, where Reistad et al. found that TBBPA can rapidly (2–4 min) activate the ERK1/2 pathway in human neurophil granulocytes and that in high concentrations TBBPA or BPA (2–12 μM) stimulate production of reactive oxygen species. Both TBBPA and high concentrations of BPA (20 μM) were shown to induce a concentration dependent increase in intracellular free calcium levels [160].

14.2. Rapid and low dose actions of BPA related to the developing CNS

Rapid low dose BPA-mediated effects on ERK signaling were characterized *in vivo* and *in vitro* in isolated primary cultures of cerebellar granule cell neurons [46,153,161]. An analysis of BPA concentration dose response by Zsarnovszky et al. revealed that BPA stimulates an inverted U-shaped curve with efficacy and potency equal to estradiol in the low dose range (10 fM to 10 nM) [46]. In the presence of pharmacological concentrations (micromolar range) of BPA and estradiol, ERK-phosphorylation was also observed, demonstrating a clear non-monotonic and biphasic dose response. Developmental and pharmacological studies of the rapid actions of estrogen and BPA on cerebellar signaling by Belcher et al. revealed a mechanism that induces cell specific activation of ERK1/2-phosphorylation that involves G-proteins, PKA and the Src-family tyrosine kinase, but not EGF receptor or the PI3kinase/AKT pathway [161]. Further, the results of experiments assessing the effects of binary mixtures of estradiol and BPA highlighted the complexity of the rapid signaling mechanism in these developing neurons [46]. These results demonstrate the ability of BPA to act as a highly potent EDC with a potential to disrupt the rapid signaling of estradiol at very low concentrations during brain development.

Numerous *in vivo* studies have shown that prenatal and neonatal exposure to low doses of BPA can disrupt normal sexual differentiation in the rodent brain [162,163]. BPA at 40 μg/kg dose can antagonize the action of estradiol in the adult rat hippocampus by blocking the stimulatory effect of estradiol on synaptogenesis [43]. Additional studies have also implicated BPA in modifying the activity of the mesolimbic dopamine system through upregulation of dopaminergic neurotransmission, an effect that in part involves increased dopamine D1 receptor expression. These endocrine disrupting activities of BPA could

potentially influence reward-seeking behaviors associated with drugs of abuse. In the pheochromocytoma PC12 cell model, high concentrations of BPA (25–150 μM) stimulate rapid release of dopamine through guanine nucleotide-binding protein and N-type calcium channels, illustrating that exposure to BPA may influence the function of dopaminergic neurons [76]. While high concentrations of BPA were used in this study, it is possible that rapid signaling mechanisms involving mobilization of intra- and extracellular calcium contribute to the impact of BPA through presynaptic influences on dopaminergic neurotransmission.

In an effort to discriminate rapid signaling actions of BPA from BPA-mediated gene expression, Yamaguchi et al. examined the effects of low dose BPA (1 pg/mL to 1 μg/mL) on the differentiation of serum-free mouse embryo (SFME) cells and astrocyte progenitor cells [40]. These investigators monitored glial fibrillary acidic protein (GFAP) expression as a marker of differentiation and found that GFAP expression was significantly increased in SFME cells in the presence of 1–100 pg/mL BPA. The BPA-induced increases in GFAP were due to activation of signal transducer and activator of transcription 3 (STAT3) and Smad1. In isolated murine midbrain astrocytes or in astrocyte/neurons co-culture Miyatake et al. demonstrated increased GFAP immunoreactivity and increased intracellular calcium in response to dopamine at BPA concentrations as low as 100 fM and 1 pM, respectively [44]. An inverted U-shaped low dose response was observed between 100 fM and 10 pM, with a second phase of effects observed at concentrations of 10 nM to 1 μM. Similar effects were not observed in response to estradiol. The investigators suggest that these results provide evidence that BPA alters dopamine responsiveness in neurones and astrocytes, which may contribute to potentiate the development of psychological dependence on drugs of abuse.

Together, the results from these CNS-related models highlight the complexity of BPA endocrine disruption effects at low physiological concentration, and the extreme importance of appropriate experimental design, such as including positive and negative controls for each experiment and doing complete dose response analysis for each end-point.

14.3. Rapid BPA effects on the endocrine pancreas

A "non-classical membrane ER" (ncmER) has been described in the endocrine pancreas that mediates the actions of BPA at concentrations as low as 0.1 nM [70]. This action is also involved in the activation of the transcription factor calcium-dependent cAMP-responsive element binding protein (CREB) where 1 nM BPA induces activation [73]. This study showed that BPA and estradiol activate CREB with the same potency as estradiol. In another study, 1 nM BPA potentiated glucose-induced calcium ion oscillations in freshly isolated islets of Langerhans [70], also implicating ncmER.

Involvement of the ncmER in modulation of glucose induced calcium signals in glucagon releasing alpha-cells of the pancreas has also been demonstrated [66]. It was shown that 1 nM BPA can suppress low-glucose induced calcium ion oscillations in alpha cells, the signal that triggers glucagon secretion, through the action of ncmER, G-proteins, and PKG. Moreover, it was demonstrated that BPA exposure rapidly increases insulin release in adult mice following a single dose of 10 μg/kg/day [67]. This effect was insensitive to an antiestrogen ICI 182,780, indicating that classic ERs may not be involved. In contrast, long-term exposure alters pancreatic insulin content and induces insulin resistance in a classic ER-dependent manner [67].

In agreement with previous findings, Adachi et al. reported that long-term exposure to 10 μg/L BPA induces insulin secretion in rat pancreatic islets following stimulation with 16.7 mM glucose. This effect is significantly suppressed by 1 μM ICI 182,780, suggesting that long-term exposure to BPA potentiates glucose-induced insulin secretion via a classic ER-mediated pathway [21]. In the same study, the maximal effective dose of estradiol for insulin secretion was also10 μg/L. However, in contrast to estradiol, BPA exhibited only long-term effects and did not induce acute insulin secretion from islets via plasma membrane ERs.

The rapid effects of BPA on intracellular calcium ion concentration have also been measured and compared to that of estradiol in breast cancer cell models (MCF-7 and MDA-MB-231). A rapid (within 1.5 min) influx of calcium was observed in response to BPA that was significant at the lowest dose tested (0.1 nM) [74]. This response was not blocked by anti-estrogens, demonstrating ER-transactivation independence of these rapid signaling effects of BPA at nanomolar concentrations. In sum, it has been shown that BPA can activate classic ERs in organelles other than the nucleus in target cells. In addition, BPA can bind and activate other estrogen binding proteins, for example ncmER and GPR30. BPA activates these receptor-mediated pathways at concentrations similar to those described for estradiol. However, further studies are needed to delineate BPA's mode of action in the pancreas, CNS and other tissues. Clearly, the studies described above are just starting to unveil the possible link between environmental estrogens, insulin resistance and glucose metabolism.

15. Immune system, allergic response and BPA exposure

It is well established that estrogens play a role in the immune system and recent research shows that BPA is capable of influencing the immune system functions [164,165]. It was demonstrated that at concentrations as low as 10 nM, BPA decreases the adherence index of rat peritoneal macrophages *in vitro* [166]. Based on the understanding that adhesion is the first step in the phagocytic process of macrophages and in antigen presentation, the authors of this study suggested that BPA may modulate immune and inflammatory responses.

To examine the effects of estradiol and BPA on chemokine production, expression of monocyte chemoattractant protein-1 (MCP-1, a member of the chemokine family) in human breast cancer cell line MCF-7 was studied [60]. MCF-7 cells produce a large quantity of MCP-1 in response to interleukin-1alpha (IL-1α). Addition of 0.1 μM BPA to MCF-7 cells inhibited MCP-1 mRNA and protein expression and electrophoretic mobility shift assay and supershift analysis revealed that treatment with 0.1–1 μM BPA diminished the IL-1α-induced complex formation, although to a lesser degree than estradiol. These results

suggest that BPA represses MCP-1 expression in a different manner than estradiol, possibly depending on the presence of cell-type specific cooperating factors and/or signaling pathways. Further studies are needed to decipher the molecular mechanism of cell type- and stimulus-specific responses.

Regulation of immunoglobulin (Ig) production by BPA and estradiol was examined by Han et al. [63]. Mouse splenocytes (BALB/c mice) were treated with 10 nM to 1 uM BPA or estradiol and the levels of IgM, IgE, IgA and IgG were measured. While IgA and IgG levels were not affected by either treatment, estradiol induced decreases in IgM and increased IgE levels. In contrast, 1 μM BPA treatment resulted in enhanced IgM production and decreased IgE levels [63].

Youn et al. examined the immune response following BPA exposure in the mouse spleen cells [58]. BPA was administrated to mice in drinking water for 4 weeks at 0.015, 1.5 and 30 mg/mL. It was found that BPA induced prolactin production in the spleen, and exposure of BPA increased the activity of splenocyte proliferation. Interestingly, the production of a strong Th-1 type cytokine (IFN-gamma) was induced, while Th-2 type (IL-4) was suppressed by BPA treatment. Based on those findings, the authors speculated that stimulation of prolactin production by estrogenic effects of BPA can affect cytokine production, thus leading to imbalanced cellular immune response. A follow-up study by Yoshino et al. demonstrated that prenatal exposure to BPA results in up-regulation of immune responses [56]. This group measured proliferative responses of spleen cells to antigen, anti-HEL IgG2a and interferon-gamma (IFN-gamma) secreted from splenic lymphocytes were also measured as indicators of T helper 1 (Th1) immune responses, while anti-HEL IgG1 and interleukin-4 (IL-4) were measured as indicators of Th2 responses. The results showed that fetal exposure to BPA was followed by significant increases in anti-HEL IgG as well as antigen-specific cell proliferation, suggesting that prenatal exposure to BPA may result in the up-regulation of immune responses in adulthood.

Goto et al. examined the effects of BPA on mouse splenocyte (BALB/c female mice) proliferation and found that at high concentrations of BPA (20 μM) enhanced Mac1+ splenocyte proliferation [64]. However, the dose used in this study is clearly outside of the physiologic range. To evaluate the effects of estradiol and BPA on the immune function, Sakazaki et al. investigated whether ERα is expressed in splenic T and B cells, and what effects these compounds have on mouse lymphocyte mitogenesis [65]. This group showed that ERα is expressed in both male and female mouse splenic lymphocytes. Next, lymphocytes from mouse spleen were exposed to estradiol or BPA under growth stimulation by lipopolysaccharide or concanavalin A as a mitogen specific for either B or T cells, respectively. It was found that exposure to estradiol (10 nM to 10 μM) or BPA at 1 μM inhibits lymphocytes mitogenesis, especially B cells. However, this group did not determine whether BPA-mediated suppression of lymphocyte proliferation was mediated by the ER-α in these cells.

Another study examined the effects of BPA at low concentrations (0.1–10 nM) on leukocyte differentiation by measuring superoxide production by differentiated HL-670 cells, a human promyelocytic cell line, in the presence of absence of tamoxifen [53]. It was found that that BPA enhances leukocyte differentiation through an ER-independent pathway. Thus, it is possible that long-term exposure to low dose BPA might significantly affect the innate immunity in humans.

The effects of BPA on the production of interleukin-4 (IL-4), a pro-inflammatory cytokine closely associated with allergic immune responses, were examined by several investigators [52,61]. Using different experimental systems, these investigations are in agreement: treatment with BPA results in enhanced production of various cytokines, including IL-4. Yamashita et al. demonstrated BPA effects on murine thymocytes *in vitro* [55]. They showed that precultured thymocytes with 10 μM BPA exhibited enhanced production of IL-3, IL-4 and interferon-gamma. Tian et al. studied the effects of BPA on the *in vitro* production of Th1 and Th2 cytokines in mesenteric lymph node cells from Trichinella spiralis (Ts)-infected mice inoculated orally with BPA [52]. They found that IL-4 production by Th2-dominant mesenteric lymph node cells from Ts-infected mice increased significantly by addition of 3 μM BPA. However, IL-5 production was not affected. This group demonstrated that the IL-4 production was increased both *in vitro* and *in vivo* by treatment with BPA, and this study suggests that BPA might cause allergic diseases by stimulating the IL-4 production by Th2 cells. It was also shown that BPA significantly enhanced IL-4 production in keyhole limpet haemocyanin (KLH)-primed CD4+ T cells in a concentration-dependent manner [61]. The lowest dose of BPA that had an effect on IL-4 production in this study was 10 μM. Furthermore, BPA enhanced the activation of IL-4 gene promoter in hybridoma EL4 T cells transiently transfected with IL-4 promoter/reporter constructs. The authors also assessed the contribution of intracellular calcium-mediated signaling to BPA-enhanced IL-4 production by pre-treating lymph node cells from the immunized mice with various inhibitors known to interfere with calcium homeostasis. BPA increased intracellular calcium ion levels by inhibiting intracellular calcium pumps. The enhancement of IL-4 production by BPA was significantly reduced by nitrendipine, which blocks calcium ion influx and by FK506, a calcineurin inhibitor, demonstrating that the enhancement of IL-4 by is mediated by a calcium/calcineurin/NF-AT signaling pathway [61].

A study by Kim et al. demonstrated that BPA may affect the regulation of the immune system by reducing nitric oxide (NO) and tumor-necrosis factor-alpha (TNF-alpha) via the inhibition of nuclear factor (NF)-kappaB transactivation. These effects of BPA are mediated through the ER, as shown in this study [48]. Peritoneal macrophages isolated from specific pathogen free-BALB/C mice and RAW 264.7 cells, a mouse macrophage cell line, were used in the study. Ten to 50 μM BPA was used. In agreement with this study, a follow-up study by Hong et al. found that BPA treatment enhanced NO production in mouse macrophages *in vitro* [62].

The involvement of endocrine disruptors in autoimmune disease was examined in the study by Yurino and colleagues. In those studies it was found that demonstrated that endocrine disruptors including DES and BPA enhance autoantibody production by B1 cells both *in vitro* and *in vivo* [59].

The production of IL-2, a cytokine that plays an important role in adaptive immune response, was examined in CD4+ Jurkat T cells in response to BPA treatment [49]. Interestingly, other endocrine-disrupting compounds (coumestrol, DDT and TCDD), but not BPA, significantly suppressed IL-2 production at the transcriptional and translational levels. These results demonstrate that BPA elicits differential responses on cytokine production and may be cell-type and stimulus specific. Recently, Yamashita et al. examined BPA effects on murine spleen cells and thymus cells *in vitro* [54]. They reported that at 0.01–1 μM BPA concentrations BPA stimulates production of IL-1, IL-6, IL-12, TNF and MCP-1. The maximum response was observed at 0.1 μM BPA. Alizadeh et al. demonstrated that low dose BPA exposure (0.1 mg/g/body weight) results in augmentation of Th1 immune response [47], thus further implicating BPA involvement in the immune system functions.

16. Conclusions and levels of confidence from the results of mechanistic *in vitro* studies

16.1. Based on existing evidence, we are confident of the following

The criterion for achieving this confidence level is that multiple independent studies had been conducted that showed the same or similar outcome.

a. BPA can act as an estrogen. Its effects are, however, cell type specific.
b. Timing (developmental stage) of exposure and exposure dose/concentration are critical.
c. When BPA binds to classic nuclear estrogen receptors and induce specific ERE binding, BPA is usually less potent than estradiol.
d. When BPA action is mediated by estrogen receptors outside the nucleus, its potency is as high as that of estradiol, ranging within the pico- and nano-molar concentrations.
e. Because of cell-type specific expression patterns and the role of varied specific co-regulatory factors, the effects of BPA might be different in individual cell types and these effects can vary depending on intrinsic and extrinsic influences.
f. BPA is not simply a SERM (selective estrogen receptor modulator).
g. BPA exerts pleiotropic cellular and tissue-type specific effects and non-monotonic dose–response at the cellular and intracellular levels at low physiologically relevant concentrations.

16.2. We consider the following to be likely but requiring confirmation

The criterion for achieving this confidence level is that significant effects have been reported, but the number of independent replications is limited. However, confidence in the findings is increased by the plausibility of the results, based on mechanistic information available from other related studies.

a. BPA exerts some of its effects by binding to the nuclear steroid receptors for estrogen (ERα and ERβ) to subsequently impact expression of estrogen-responsive gene products via the EREs. Thus, cell types that express specific ERs and co-modulatory elements can be especially sensitive to the effects of BPA.
b. BPA exerts some of its effects by binding to nuclear steroid receptors for androgens—the (AR). BPA can act as an antagonist for the wild type AR in some tissues.
c. Ligand-binding domain somatic mutants of ARs are important in prostate cancer: they can be activated by low dose (chronic exposure) of BPA.
d. In cells from the male reproductive system BPA impacts the biosynthesis of steroids which results in altered steroid hormone concentrations.
e. Compared to the impact of brominated BPA derivatives, these studies point toward BPA at physiological concentrations ($\leq 1 \times 10^{-7}$ M) has a relatively minor influence on the TH system.

16.3. Research on BPA suggests several broad themes that should be pursued in the future

a. Because of the apparently complex nature of BPA action on the TH-signaling systems studied to date, additional studies are required in order to develop a mechanistic understanding of BPA's influence of TH-signaling, which has an important impact on the development of wildlife and humans.
b. Increase our knowledge of the physiological/biological relevance of BPA binding to the ER, AR and TR.
c. Investigate the mechanisms responsible for inverted U dose response.
d. We need to develop sensitive cell model systems that detect altered patterns of differentiation and physiologically relevant responses, avoiding systems in which regulators are over-expressed.
e. Identify other BPA-binding proteins and investigate GPR-30 actions in specific cell types upon exposure to endocrine disrupting compounds.
f. Define additive and synergistic effects of BPA and endogenous hormones and other endocrine disruptors.
g. While there are numerous *in vivo* studies demonstrating impact on the nervous system upon early exposures to BPA, much additional research is necessary in order to assign specific molecular mechanism through which BPA acts to impact specific neuronal and glial populations in the developing and mature CNS.
h. Explore low dose BPA exposure on the immune system.
i. Molecular mechanism(s) of acute versus chronic exposure to BPA need to be distinguished and deciphered.
j. Define cell and tissue-type sensitivity and windows of susceptibility to BPA across the life span of experimental animals and humans.

References

[1] Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL. Urinary concentrations of bisphenol A and 4-nonylphenol in

a human reference population. Environ Health Perspect 2005;113(4): 391–5.
[2] Kang JH, Kondo F, Katayama Y. Human exposure to bisphenol A. Toxicology 2006;226(2/3):79–89.
[3] Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, et al. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect 1996;104(Suppl 4):715–40.
[4] Welshons WV, Nagel SC, vom Saal FS. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 2006;147(6 Suppl):S56–69.
[5] Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, et al. Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol 1998;142(1/2):203–14.
[6] Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997;138(3):863–70.
[7] Matthews JB, Twomey K, Zacharewski TR. In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. Chem Res Toxicol 2001;14(2):149–57.
[8] Routledge EJ, White R, Parker MG, Sumpter JP. Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. J Biol Chem 2000;275(46):35986–93.
[9] Recchia AG, Vivacqua A, Gabriele S, Carpino A, Fasanella G, Rago V, et al. Xenoestrogens and the induction of proliferative effects in breast cancer cells via direct activation of oestrogen receptor alpha. Food Addit Contam 2004;21(2):134–44.
[10] Vivacqua A, Recchia AG, Fasanella G, Gabriele S, Carpino A, Rago V, et al. The food contaminants bisphenol A and 4-nonylphenol act as agonists for estrogen receptor alpha in MCF7 breast cancer cells. Endocrine 2003;22(3):275–84.
[11] Seidlova-Wuttke D, Jarry H, Wuttke W. Pure estrogenic effect of benzophenone-2 (BP2) but not of bisphenol A (BPA) and dibutylphtalate (DBP) in uterus, vagina and bone. Toxicology 2004;205(1/2):103–12.
[12] Satoh K, Ohyama K, Aoki N, Iida M, Nagai F. Study on anti-androgenic effects of bisphenol a diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives using cells stably transfected with human androgen receptor, AR-EcoScreen. Food Chem Toxicol 2004;42(6):983–93.
[13] Hall JM, Korach KS. Analysis of the molecular mechanisms of human estrogen receptors alpha and beta reveals differential specificity in target promoter regulation by xenoestrogens. J Biol Chem 2002;277(46):44455–61.
[14] Pennie WD, Aldridge TC, Brooks AN. Differential activation by xenoestrogens of ER alpha and ER beta when linked to different response elements. J Endocrinol 1998;158(3):R11–4.
[15] Olsen CM, Meussen-Elholm ET, Samuelsen M, Holme JA, Hongslo JK. Effects of the environmental oestrogens bisphenol A, tetrachlorobisphenol A, tetrabromobisphenol A, 4-hydroxybiphenyl and 4,4′-dihydroxybiphenyl on oestrogen receptor binding, cell proliferation and regulation of oestrogen sensitive proteins in the human breast cancer cell line MCF-7. Pharmacol Toxicol 2003;92(4):180–8.
[16] Kurosawa T, Hiroi H, Tsutsumi O, Ishikawa T, Osuga Y, Fujiwara T, et al. The activity of bisphenol A depends on both the estrogen receptor subtype and the cell type. Endocr J 2002;49(4):465–71.
[17] Ackermann GE, Brombacher E, Fent K. Development of a fish reporter gene system for the assessment of estrogenic compounds and sewage treatment plant effluents. Environ Toxicol Chem 2002;21(9):1864–75.
[18] Mueller SO, Kling M, Arifin Firzani P, Mecky A, Duranti E, Shields-Botella J, et al. Activation of estrogen receptor alpha and ERbeta by 4-methylbenzylidene-camphor in human and rat cells: comparison with phyto- and xenoestrogens. Toxicol Lett 2003;142(1/2):89–101.
[19] Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ Health Perspect 1997;105(1):70–6.
[20] Watson CS, Campbell CH, Gametchu B. Membrane oestrogen receptors on rat pituitary tumour cells: immuno-identification and responses to oestradiol and xenoestrogens. Exp Physiol 1999;84(6):1013–22.
[21] Adachi T, Yasuda K, Mori C, Yoshinaga M, Aoki N, Tsujimoto G, et al. Promoting insulin secretion in pancreatic islets by means of bisphenol A and nonylphenol via intracellular estrogen receptors. Food Chem Toxicol 2005;43(5):713–9.
[22] Roy P, Salminen H, Koskimies P, Simola J, Smeds A, Saukko P, et al. Screening of some anti-androgenic endocrine disruptors using a recombinant cell-based in vitro bioassay. J Steroid Biochem Mol Biol 2004;88(2):157–66.
[23] Sohoni P, Sumpter JP. Several environmental oestrogens are also anti-androgens. J Endocrinol 1998;158(3):327–39.
[24] Sun H, Xu LC, Chen JF, Song L, Wang XR. Effect of bisphenol A, tetrachlorobisphenol A and pentachlorophenol on the transcriptional activities of androgen receptor-mediated reporter gene. Food Chem Toxicol 2006;44(11):1916–21.
[25] Lee MS, Hyun SH, Lee CK, Im KS, Hwang IT, Lee HJ. Impact of xenoestrogens on the growth of human endometrial epithelial cells in a primary culture system. Fertil Steril 2003;79(6):1464–5.
[26] Wetherill YB, Petre CE, Monk KR, Puga A, Knudsen KE. The xenoestrogen bisphenol A induces inappropriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells. Mol Cancer Ther 2002;1(7):515–24.
[27] Xu LC, Sun H, Chen JF, Bian Q, Qian J, Song L, et al. Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro. Toxicology 2005;216(2/3):197–203.
[28] Wetherill YB, Fisher NL, Staubach A, Danielsen M, de Vere White RW, Knudsen KE. Xenoestrogen action in prostate cancer: pleiotropic effects dependent on androgen receptor status. Cancer Res 2005;65(1):54–65.
[29] Lee HJ, Chattopadhyay S, Gong EY, Ahn RS, Lee K. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicol Sci 2003;75(1):40–6.
[30] Ghisari M, Bonefeld-Jorgensen EC. Impact of environmental chemicals on the thyroid hormone function in pituitary rat GH3 cells. Mol Cell Endocrinol 2005;244(1/2):31–41.
[31] Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MH, Andersson PL, et al. In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol Sci 2006;92(1):157–73.
[32] Iwamuro S, Yamada M, Kato M, Kikuyama S. Effects of bisphenol A on thyroid hormone-dependent up-regulation of thyroid hormone receptor alpha and beta and down-regulation of retinoid X receptor gamma in Xenopus tail culture. Life Sci 2006.
[33] Kitamura S, Jinno N, Ohta S, Kuroki H, Fujimoto N. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. Biochem Biophys Res Commun 2002;293(1):554–9.
[34] Kitamura S, Kato T, Iida M, Jinno N, Suzuki T, Ohta S, et al. Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis. Life Sci 2005;76(14):1589–601.
[35] Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K, et al. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 2005;84(2):249–59.
[36] Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, et al. Thyroid hormone action is disrupted by bisphenol A as an antagonist. J Clin Endocrinol Metab 2002;87(11):5185–90.
[37] Zoeller RT, Bansal R, Parris C. Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinology 2005;146(2):607–12.
[38] Zoeller RT, Crofton KM. Thyroid hormone action in fetal brain development and potential for disruption by environmental chemicals. Neurotoxicology 2000;21(6):935–45.
[39] Zoeller RT. Environmental chemicals as thyroid hormone analogues: new studies indicate that thyroid hormone receptors are targets of industrial chemicals? Mol Cell Endocrinol 2005;242(1/2):10–5.
[40] Yamaguchi H, Zhu J, Yu T, Sasaki K, Umetsu H, Kidachi Y, et al. Low-level bisphenol A increases production of glial fibrillary acidic protein