

Table 1 (Continued)

Reference	Species	Model	Endpoints	Exposure: vehicle	Exposure time	LOEC (M) ( <sup>a</sup> P < 0.05)	Doses tested (M) ( <sup>a</sup> 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 <sup>-5</sup> μ	2.5 × 10 <sup>-6</sup> , 5 × 10 <sup>-6</sup> , 1 × 10 <sup>-5</sup> , 2 × 10 <sup>-5</sup> μ		
Han et al., 2002 [63]	Mice BALB/c	Male primary splenocytes	Immunooglobulin production (ELISA)	Culture medium, <0.1% ETOH	7 days	10 <sup>-5</sup> (IgE: IgM)	10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> , 10 <sup>-3</sup>		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	4 h	nd	4.4 × 10 <sup>-5</sup> μ (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)	DMSO/control oil	6, 24 h	10 <sup>-5</sup> μ	10 <sup>-6</sup> , 10 <sup>-5</sup> μ, 5 × 10 <sup>-5</sup> μ, 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; EL-4)	PMA/KLH-primed IL-4-production	Culture medium, Not indicated	2, 4 days	10 <sup>-5</sup> μ	10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> μ, 5 × 10 <sup>-5</sup> μ, 10 <sup>-4</sup> μ	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 <sup>-9</sup> , 10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup>		Comestrol; TCDD; DDT
Reisand et al., 2005 [160]	Human	Primary neutrophil granulocytes	Reactive oxygen species, Intracellular Ca <sup>2+</sup> (secondary endpoints)	HEPES-buffered salt solution, ≤0.1% ETOH	60 min, 240 s	2 × 10 <sup>-6</sup> μ	2 × 10 <sup>-6</sup> μ, 6 × 10 <sup>-6</sup> μ, 1.2 × 10 <sup>-5</sup> μ, 2 × 10 <sup>-5</sup> μ		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 <sup>-6</sup> μ	3 × 10 <sup>-8</sup> , 3 × 10 <sup>-7</sup> , 3 × 10 <sup>-6</sup> μ, 3 × 10 <sup>-5</sup> μ, 3 × 10 <sup>-3</sup> × 10 <sup>-6</sup>		E2; genistein; coumestrol; α-zeranol; progesterone; cholesterol
Sawai et al., 2003 [51]	Mice C57BL/6j	Primary splenic mononuclear cells HL-60	ConA induced IFN-γ secretion (ELISA)	Culture medium, Not indicated	24 h	5 × 10 <sup>-8</sup> μ	5 × 10 <sup>-10</sup> , 5 × 10 <sup>-8</sup> μ, 5 × 10 <sup>-6</sup> μ		
Watanabe et al., 2003 [53]	Human		O <sub>2</sub> <sup>-</sup> production, CD18 expression	Culture medium, DMSO	6 days	10 <sup>-10</sup> μ	10 <sup>-12</sup> , 10 <sup>-10</sup> , 10 <sup>-9</sup> μ, 10 <sup>-8</sup>	4-OH Tamoxifen	
Liver models									
Jeong et al., 2000 [83]	Mice	Hepn-1/c17 (hepatoma)	Cyp1a-1 activity, Cyp1a-1 mRNA expression (RT-PCR)	Culture medium, DMSO <0.2%	18 h, 6 h	10 <sup>-5</sup> μ	10 <sup>-6</sup> , 10 <sup>-5</sup> μ, 5 × 10 <sup>-5</sup> μ	4-OH Tamoxifen	TCDD
Kuho et al., 2004 [78]	Human	Hep3B (hepatoma)	Inhibition of hypoxic response, Erythropoietin, HIF-1α expression	Culture medium, Not indicated	6 h	10 <sup>-4</sup>	5 × 10 <sup>-5</sup> , 10 <sup>-4</sup> , 5 × 10 <sup>-4</sup>	CoCl <sub>2</sub> 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 <sup>-5</sup>	4.5 × 10 <sup>-5</sup>		BPA-methacrylate; <i>o,p'</i> -DDT
Iida et al., 2003 [175]	Rat Wistar	Primary Sertoli cells	Viability/apoptosis	Culture media	<12–48 h	1.5 × 10 <sup>-4</sup> μ	5 × 10 <sup>-5</sup> , 10 <sup>-4</sup> , 1.5 × 10 <sup>-4</sup> μ, 2 × 10 <sup>-4</sup> μ, 3 × 10 <sup>-4</sup> μ, 10 <sup>-3</sup> , 10 <sup>-2</sup> , 10 <sup>-1</sup>		
Lee et al., 2003 [29]	S. cerevisiae, Human, Mice	HeLa, HepG2, L5p-1 (Sertoli cell line)	2-hybrid AR/ASCI, GFP-AR transfection, Inhibition of ARE-transcription (luciferase)	Culture medium, ETOH	3 h, 24 h, 24 h	10 <sup>-6</sup>	10 <sup>-7</sup> , 10 <sup>-6</sup> μ, 10 <sup>-5</sup> μ, 10 <sup>-4</sup> μ		E2; NP; cyproterone
Nikula et al., 1999 [135]	Mice	mLTC-1 (Leydig tumor cell line)	cAMP, progesterone production	Culture medium, ETOH	48 h	10 <sup>-7</sup> μ	10 <sup>-7</sup> , 10 <sup>-6</sup> μ, 10 <sup>-5</sup> μ, 10 <sup>-4</sup> μ		E2; <i>tert</i> -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 <sup>-8</sup>	10 <sup>-9</sup> , 10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup>	H89; PD98059; GFX; wortmannin	BPE; BPF; BPM; BPF; BPS; BPZ
Tahuchi et al., 2006 [176]	Mice	TTE3 Sertoli cells (testis)	mRNA expression	Culture media, 0.1% DMSO	3, 6, 12 h	2 × 10 <sup>-4</sup> μ	2 × 10 <sup>-4</sup> μ		
Wetherill et al., 2002 [26]	Human	LNCaP (prostate)	Cell proliferation, AR-translocation	Culture medium, 0.1% ETOH	72 h, 2, 7 h	10 <sup>-9</sup> μ, 10 <sup>-10</sup> μ	10 <sup>-10</sup> , 10 <sup>-9</sup> μ, 10 <sup>-8</sup> , 10 <sup>-7</sup>	Bicalutamide	DHT
Wetherill et al., 2005 [28]	Human	LNCaP (prostate)	PSA, AR expression, AR transactivation	Culture medium, 0.1% ETOH	48 h	10 <sup>-9</sup>	10 <sup>-9</sup> , 10 <sup>-5</sup>	Bicalutamide	DHT
Saeh et al., 2004 [12]	Hamster	CHO-K1 (ARE <sup>-</sup> luciferase)	AR/ARE <sup>-</sup> luc expression (± DHT), Cell proliferation	Culture medium, DMSO, ≤0.1% ETOH	16–24 h	10 <sup>-6</sup> , 10 <sup>-7</sup>	10 <sup>-11</sup> , 10 <sup>-10</sup> , 10 <sup>-9</sup> , 10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-2</sup> , 10 <sup>-5</sup>		BADGE; BADGE-3H; O; BADGE-3HCl; BPF; BFDGE; BFDGE-3H; O; Mitholesterone
Nervous system models									
Miyatake et al., 2006 [44]	Mice ICR	Primary culture midbrain astrocytes & neurons/glia	GFP/Neu-N immunoreactivity, [Ca <sup>2+</sup> ] <sub>i</sub>	Culture medium, Not indicated	24 h	10 <sup>-13</sup> μ	10 <sup>-14</sup> , 10 <sup>-13</sup> μ, 10 <sup>-12</sup> μ, 10 <sup>-11</sup> μ, 10 <sup>-10</sup> , 10 <sup>-10</sup> , 10 <sup>-9</sup> , 10 <sup>-8</sup> μ	ICI 182,780; mifepristone; flutamide	E2

Table 1 (Continued)

Reference	Species	Model	Endpoint	Exposure: vehicle	Exposure time	LOEC (M) (*P < 0.05)	Doses tested (M) (*P < 0.05)	Inhibitors	Other chemicals tested
Saito et al. 2004 [177]	Mice ICR	Primary oligodendrocyte precursor cells	T3-induced differentiation	Culture media, Not indicated	3 days	10 <sup>-5</sup>	10 <sup>-5</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup>	ICI 182,780	E2, 17 $\alpha$ -EE, DES, p-NP
Sato et al. 2002 [178]	Rat Wistar	Hippocampus organotypic primary culture	Glutamate-induced cell death (CA3)	Culture medium	24 h	10 <sup>-12</sup> <sub>a</sub>	10 <sup>-12</sup> <sub>a</sub>		
Yamaguchi et al. 2006 [40]	Mice	SFME cells	NMDA-receptor expression; Spine & mossy fiber density; LIF/BNP-2 induced astrocyte differentiation	$\leq 0.1\%$ ETOH Culture medium, Not indicated	48 h	4.4 $\times$ 10 <sup>-12</sup> <sub>b</sub>	4.4 $\times$ 10 <sup>-13</sup> , 4.4 $\times$ 10 <sup>-12</sup> <sub>a</sub> , 4.4 $\times$ 10 <sup>-11</sup> <sub>a</sub> , 4.4 $\times$ 10 <sup>-10</sup> <sub>a</sub> , 4.4 $\times$ 10 <sup>-9</sup> , 4.4 $\times$ 10 <sup>-8</sup> , 4.4 $\times$ 10 <sup>-7</sup> , 4.4 $\times$ 10 <sup>-6</sup> , 4.4 $\times$ 10 <sup>-5</sup> , 4.4 $\times$ 10 <sup>-4</sup>	4-OH Tamoxifen	
Yoneda et al. 2003 [76]	Rat	PC12 (adrenal)	Dopamine secretion	Culture medium, $\leq 0.1\%$ DMSO	10 min	2.5 $\times$ 10 <sup>-6</sup> <sub>a</sub>	2.5 $\times$ 10 <sup>-6</sup> <sub>a</sub> , 5 $\times$ 10 <sup>-6</sup> <sub>a</sub> , 1 $\times$ 10 <sup>-5</sup> <sub>a</sub> , 1.5 $\times$ 10 <sup>-5</sup> <sub>a</sub>	Diltiazem; clogylin; pargyline; $\omega$ -ligatoxin; $\omega$ -conotoxin; H7; H-89; tyranoline; ruthenium red; Rp-cAMPS; GDP $\beta$ s; U0128	E2
Zarnovszky et al. 2005 [46]	Rat Sprague-Dawley	Developing cerebellar neurons (male & female)	ERK1/2 phosphorylation	Culture medium, artificial cerebrospinal fluid, DMSO	5 min	10 <sup>-12</sup> <sub>a</sub>	10 <sup>-12</sup> <sub>a</sub> , 10 <sup>-11</sup> <sub>a</sub> , 10 <sup>-10</sup> <sub>a</sub> , 10 <sup>-9</sup> , 10 <sup>-8</sup> , 10 <sup>-7</sup> <sub>a</sub> , 10 <sup>-6</sup> <sub>a</sub> , 10 <sup>-4</sup>	Actinomycin-D; ICI 182,780	E2, NP
Pancreatic models									
Atachi et al. 2005 [21]	Rat Wistar	Male; primary pancreatic islets	Insulin release-RIA	Krebs-Ringer bicarbonate buffer	60 min, 24 h	nd, 4.4 $\times$ 10 <sup>-8</sup> <sub>a</sub>	4.4 $\times$ 10 <sup>-10</sup> , 4.4 $\times$ 10 <sup>-9</sup> , 4.4 $\times$ 10 <sup>-8</sup> <sub>a</sub> , 4.4 $\times$ 10 <sup>-7</sup> <sub>a</sub> , 4.4 $\times$ 10 <sup>-6</sup> <sub>a</sub> , 4.4 $\times$ 10 <sup>-5</sup> <sub>a</sub> , 10 <sup>-4</sup> <sub>a</sub>		
Alonso-Magdalena et al. 2005 [66]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Intracellular Ca <sup>2+</sup>	-16.7 mM glucose Modified Ringers-5 or 0.5 mM glucose, DMSO	5 min	10 <sup>-9</sup> <sub>a</sub>	10 <sup>-9</sup> <sub>a</sub>	ICI 182,780; KT-5823;	E2, E2-HRP, DES; DDT <i>o,p'</i> -DDT
Alonso-Magdalena et al. 2006 [67]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Insulin release-RIA; insulin content	<i>In vivo</i> sc injection; iocopherol-stripped corn oil	4–8 min	10 <sup>-10</sup>	10 <sup>-10</sup> , 10 <sup>-11</sup> , 10 <sup>-9</sup> , 10 <sup>-7</sup>		
Nadal et al. 2000 [70]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Fluo-3 AM [Ca <sup>2+</sup> ] <sub>i</sub>	Perfusion buffer -8 mM glucose	0–15 min	10 <sup>-9</sup>	10 <sup>-9</sup>	ICI 182,780	E2, E2-HRP
Quesada et al. 2002 [72]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Intracellular Ca <sup>2+</sup> ; Phospho-CREB-IR	Perfusion buffer	0–15 min	10 <sup>-9</sup>	10 <sup>-9</sup>		
Pituitary models									
Bulayeva et al. 2004 [69]	Rat	GH3/B6/F10 (ER $\alpha$ -positive) GH3	ERK1/2 phosphorylation immunoreactivity	Culture medium; ETOH	3–30 min	nd	10 <sup>-9</sup>		E2, NP; endosulfan; DDE; dieldrin; conestrol
Chisari et al. 2005 [30]	Rat	GH3	Cell proliferation (T-screen)	Culture medium	6 days	10 <sup>-7</sup> <sub>a</sub>	10 <sup>-8</sup> , 10 <sup>-7</sup> <sub>a</sub> , 10 <sup>-6</sup> <sub>a</sub> , 10 <sup>-5</sup> <sub>a</sub>	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-PCB 121
Kitamura et al. 2002 [33]	Rat	GH3	Cell proliferation	Culture medium, ETOH	7 days	10 <sup>-6</sup> <sub>a</sub>	10 <sup>-7</sup> , 10 <sup>-6</sup> <sub>a</sub> , 10 <sup>-5</sup> <sub>a</sub>	4-OH Tamoxifen	T3; E2; TBBPA; TC8PA
Kitamura et al. 2005 [35]	Hunan. Mice. Rat	MCF7, NIH3T3, GH3	ERE $\alpha$ -luciferase, ARE $\alpha$ -luciferase, GH-induction	Culture medium, ETOH	24 h, 24 h, 2 days	10 <sup>-7</sup> <sub>a</sub> , 10 <sup>-6</sup> <sub>a</sub> , NS	10 <sup>-8</sup> , 10 <sup>-7</sup> <sub>a</sub> , 10 <sup>-6</sup> <sub>a</sub> , 10 <sup>-5</sup> <sub>a</sub> , 10 <sup>-4</sup> <sub>a</sub>	4-OH Tamoxifen	E2; various BPA-related compounds
Steinmetz et al. 1997 [157]	Rat	GH3	Prolactin release, Cell proliferation	Culture media, $\leq 0.001\%$ ETOH	1–8 days, 8, 24 h	10 <sup>-6</sup> , 10 <sup>-9</sup>	10 <sup>-6</sup> , 10 <sup>-9</sup>		E2
Watson et al. 2005 [72]	Rat	GH3/B6/F10 GH3/B6/D9	Prolactin-luciferase	Ringer's solution, Culture medium	1, 3 min	10 <sup>-12</sup>	10 <sup>-12</sup> , 10 <sup>-10</sup> <sub>a</sub> , 10 <sup>-9</sup> <sub>a</sub> , 10 <sup>-8</sup> <sub>a</sub> , 10 <sup>-7</sup>		Conestrol; DDE; DES; dieldrin; endosulfan; p-NP
Wozniak et al. 2005 [77]	Rat	GH3/B6/F10 GH3/B6/D9	Intracellular Ca <sup>2+</sup> ; Prolactin release	Ringer's solution, Culture medium	0–20 min, 1–15 min	10 <sup>-12</sup> <sub>a</sub>	10 <sup>-12</sup> <sub>a</sub> , 10 <sup>-10</sup> <sub>a</sub> , 10 <sup>-9</sup> <sub>a</sub> , 10 <sup>-8</sup> <sub>a</sub> , 10 <sup>-7</sup> <sub>a</sub> , 10 <sup>-6</sup> <sub>a</sub> , 10 <sup>-5</sup> <sub>a</sub> , 10 <sup>-4</sup> <sub>a</sub> , 10 <sup>-3</sup> <sub>a</sub>	Nifedipine	Conestrol; 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 ER $\alpha$  and ER $\beta$ , and has a higher affinity for ER $\beta$  in target cells [6–8]. Kuiper et al. reported a  $K_i$  for BPA of 195 and 35 nM at ER $\alpha$  and ER $\beta$ , respectively [6]. It was also found that the binding affinity relative to 17 $\beta$ -estradiol for BPA at ER $\beta$  was 6.6-fold higher than at ER $\alpha$ ; 0.33 and 0.05, respectively [6]. Satoh et al. also showed that BPA could bind to human ER $\alpha$  and reported an  $IC_{50}$  (the concentration of chemical required to reduce specific 17 $\beta$ -estradiol binding by 50%) of 7.8  $\mu$ M [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 ER $\alpha$ , with BPA interacting differently within the ligand-binding domain [5]. These findings reveal that the interactions of BPA and 17 $\beta$ -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 ER $\alpha$  and ER $\beta$  [8]. Specifically, the BPA/ER $\beta$  receptor/ligand complex showed over 500-fold greater potency than the BPA/ER $\alpha$  complex in recruiting the co-activator TIF2. Moreover, the enhanced (relative to ER $\alpha$ ) ability of ER $\beta$  to recruit co-activators in the presence of BPA was consistent with a greater capacity of ER $\beta$  to potentiate reporter gene activity in transiently transfected HeLa cells expressing ER $\beta$  and TIF2 over a concentration range of 1–100  $\mu$ M [8]. A higher relative binding affinity (RBA) for BPA at ER $\beta$  was also observed (estradiol RBA for BPA at ER $\beta$  was 0.75 versus 0.073 for ER $\alpha$ ). Based on those data it was concluded that ligand-dependent differences in the ability of ER $\alpha$  and ER $\beta$  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 ER $\alpha$  and ER $\beta$  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 ER $\beta$  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 17 $\beta$ -estradiol, BPA blocks the ubiquitination and degradation of ER $\beta$ , suggesting that BPA may affect ER $\beta$ -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 ER $\alpha$  and ER $\beta$  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  $\mu$ M and 17 $\beta$ -estradiol (1 nM to 1  $\mu$ M) in COS-1 cells transiently transfected with either vitellogenin ERE or LH $\beta$  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 ER $\alpha$  and ER $\beta$  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 ER $\alpha$  or ER $\beta$  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 ER $\beta$  was significantly influenced by the nature of the promoter utilized. In particular, BPA displayed remarkably high potency on pS2 promoter through ER $\beta$ , with higher ER $\alpha$ -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 ER $\beta$ , and has mixed agonist and antagonist activity in some types of cells via ER $\alpha$  [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, TGF $\beta$ -2, and STAT inhibitor 2 in MCF-7 breast cancer cells stably expressing HA-tagged ER $\alpha$  [98]. When HOXC6 mRNA expression was examined by quantitative RT-PCR, three independent ER $\alpha$ -HA cell cultures showed down-regulation (2.2–2.8-fold) following 3 h treatment with 1  $\mu$ M 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 Singleton's study, Buterin et al. demonstrated that exposure of MCF-7 and T47D breast

cancer cells to pharmacological concentrations of BPA (10  $\mu$ M) 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  $\mu$ M) 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  $\beta$ -galactosidase ( $\beta$ -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  $\mu$ g/kg (40% efficacy as compared to DES-maximum) and exhibited 20% of DES-induced maximum response at a low dose of 25  $\mu$ g/kg; however, BPA had little uterotrophic 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  $\mu$ g/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  $\mu$ g/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 ER $\alpha$ , ER $\beta$  and C3 transcripts in the uterus. It was found that estradiol had no effect on ER $\alpha$  mRNA, decreased ER $\beta$  mRNA, and greatly increased C3 mRNA expression. Interestingly, these effects were not mimicked by BPA. In fact, BPA exposure increased ER $\alpha$ , did not effect ER $\beta$ , and decreased C3 mRNA expression [11]. Treatment with both 37 and 370  $\mu$ g/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 ER $\alpha$  or ER $\beta$ . 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 ER $\alpha$  and ER $\beta$ , whereas the granulosa cells express predominantly ER $\beta$ . Accordingly, BPA has the potential to affect both theca and granulosa cells, however the degree to which the differential expression of ER $\alpha$  and ER $\beta$ , 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  $\mu$ M BPA [25]. Granulosa cells express ERs and are essential for ovarian folliculogenesis 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 17 $\beta$ -estradiol (1  $\mu$ g/mL) exert antiapoptotic effect on ovarian granulosa cells [113,114]. Based on previous findings demonstrating that BPA could partially antagonize the effects of 17 $\beta$ -estradiol at ER $\alpha$ , but not ER $\beta$  [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  $\mu$ g/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 5 $\alpha$ -dihydrotestosterone (DHT) for binding at the androgen receptor (AR) with an IC<sub>50</sub> (the concentration of chemical required to reduce the specific DHT binding by 50%) value of 2.14  $\mu$ M [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<sub>50</sub> value of  $\sim$ 0.8  $\mu$ M; 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 derivatives 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<sub>50</sub> value for AR was 7.9  $\mu$ M, and complete inhibition appeared at 19  $\mu$ M [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<sub>50</sub> = 19.6  $\mu$ M [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  $\mu$ g/kg/day) and estradiol (0.02–2  $\mu$ g/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  $\mu$ g/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  $\mu$ M 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 17 $\alpha$ -monooxygenase (17 $\alpha$ -hydroxylase/17–20 lyase) [134]. Further, post-natal exposure to BPA decreased serum 17 $\beta$ -estradiol levels in Long-Evans rats exposed to 2.4, 10, or 100  $\mu$ g/kg/day of BPA by inhibiting Leydig cell aromatase activity and exposure to lowest and environmentally relevant BPA dose (2.4  $\mu$ g/kg/day) decreased the mRNA levels of luteinizing hormone (LH)- $\beta$  and increased

ER $\beta$  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 steroidogenesis 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  $\mu$ M), 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  $\mu$ g/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 ER $\alpha$  [141]. A study in 2002 by Kitamura et al. showed that BPA, tetrabromo- and tetrachloro-BPA (TBBPA and TCBPA, respectively) could displace thyroid hormone  $^{125}\text{I-T}_3$  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 TR $\beta$ , TR $\alpha$  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  $\mu\text{M}$  and 1–100  $\mu\text{M}$ , 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 T $_3$  in an additive way. In contrast, Moriyama et al., who utilized rat liver nuclei that express both the TR $\alpha$  and TR $\beta$ , 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 TR $\alpha$  or TR $\beta$  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  $\mu\text{M}$  inhibited both the TR $\alpha$ - and TR $\beta$ -stimulated response induced by thyroid hormone T $_3$ . In the second approach, investigators co-transfected full length TR $\alpha$  and TR $\beta$  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 TSH $\alpha$  TRE. They found that in the presence of both TR $\alpha$  and TR $\beta$ , 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 TSH $\alpha$  TRE. Because the TSH $\alpha$  TRE is a negative response element, the authors speculated that BPA stabilized the interaction between TR $\alpha$ 1 or TR $\beta$ 1 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 TSH $\alpha$  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  $\mu\text{M}$ ) and TBBPA (5  $\mu\text{M}$ ) 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 TR $\alpha$  or TR $\beta$  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  $\mu\text{M}$ , but this action depended upon the compound and the TR isoform. In general, TBBPA was more potent than TCBPA, and TR $\alpha$  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  $\mu\text{M}$ . 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  $\mu\text{M}$ ) 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<sub>3</sub>-receptors. In this proliferation assay, resazurine 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<sub>3</sub>.

Taken together, *in vitro* studies demonstrate that BPA binds to both the TR $\alpha$  and TR $\beta$  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 inhibits T<sub>4</sub>-accelerated metamorphosis [146]. These investigators also showed *in vitro* that T<sub>4</sub>-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 receptor/thyroid receptor. This finding is consistent with those of Kitamura et al. where T<sub>3</sub>-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<sub>3</sub> agonist and antagonist activities at submicromolar concentrations in a *Xenopus laevis* cell line and in tadpoles [147]. These compounds competed with T<sub>3</sub> binding to  $\alpha$ TTR and  $\alpha$ TR *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<sub>50</sub> 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<sub>3</sub> 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<sub>3</sub> [148].

Iwamuro et al. investigated the effects of BPA on the expression of TR- $\alpha$  and TR- $\beta$ , 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<sub>3</sub> [32]. In the absence of T<sub>3</sub>, BPA at all concentrations examined did not show remarkable effects on tail length, but blocked T<sub>3</sub>-induced tail resorption in a concentration-dependent manner. Changes in expression of TR $\alpha$  and TR $\beta$  mRNAs from the tail specimens (measured by RT-PCR) indicated that low BPA dose (0.1  $\mu$ M) showed an apparent antagonistic effect towards the receptors and reduced mRNA levels. When administered together with T<sub>3</sub>, the antagonistic effects of BPA were more pronounced. Auto-induction of both TR $\alpha$  and TR $\beta$  genes by T<sub>3</sub> was inhibited by BPA, but the effect was less marked on TR $\alpha$  than on TR $\beta$ . Gene expression of RXR $\gamma$ , a partner for heterodimer formation of TRs, was moderately suppressed either by T<sub>3</sub> 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<sub>4</sub> in rat pups, but also increased RC3 expression in the dentate gyrus. Their interpretation was that BPA acted as a selective TR $\beta$  antagonist, causing an increase in serum T<sub>4</sub>. However, because RC3 expression is driven by the TR $\alpha$  in the dentate gyrus, these authors speculated that BPA was not exerting the same action on the TR $\alpha$ . In contrast, Nakamura et al., who studied whether prenatal exposure to low-doses of BPA by injection of pregnant mice with 20  $\mu$ g/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 TR $\alpha$  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 TR $\alpha$ , but not TR $\beta$ , 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<sub>50</sub> 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  $17\beta$ -estradiol) equals 2.83 nM [79]. It is notable that the RBA for BPA at GPR30 is higher than that observed for  $ER\alpha$  or  $ER\beta$ . 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  $\mu$ M 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  $ER\alpha$  epitope were non-responsive, these rapid signaling effects of BPA were associated with expression of an

extracellular accessible  $ER\alpha$ -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]. These 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 neutrophil granulocytes and that in high concentrations TBBPA or BPA (2–12  $\mu$ M) stimulate production of reactive oxygen species. Both TBBPA and high concentrations of BPA (20  $\mu$ M) 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  $\mu$ g/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  $\mu\text{M}$ ) 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  $\mu\text{g/mL}$ ) 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  $\mu\text{M}$ . 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  $\mu\text{g/kg/day}$  [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  $\mu\text{g/L}$  BPA induces insulin secretion in rat pancreatic islets following stimulation with 16.7 mM glucose. This effect is significantly suppressed by 1  $\mu\text{M}$  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 also 10  $\mu\text{g/L}$ . 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-1 alpha (IL-1 $\alpha$ ). Addition of 0.1  $\mu\text{M}$  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  $\mu\text{M}$  BPA diminished the IL-1 $\alpha$ -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  $\mu$ M 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  $\mu$ M 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 administered 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  $\mu$ M) 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 ER $\alpha$  is expressed in splenic T and B cells, and what effects these compounds have on mouse lymphocyte mitogenesis [65]. This group showed that ER $\alpha$  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  $\mu$ M) or BPA at 1  $\mu$ M inhibits lymphocytes mitogenesis, especially B cells. However, this group did not determine whether BPA-mediated suppression of lymphocyte proliferation was mediated by the ER- $\alpha$  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  $\mu$ M 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  $\mu$ M 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  $\mu$ M. 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)- $\kappa$ B 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  $\mu$ M 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  $\mu$ M BPA concentrations BPA stimulates production of IL-1, IL-6, IL-12, TNF and MCP-1. The maximum response was observed at 0.1  $\mu$ M 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 (ER $\alpha$  and ER $\beta$ ) 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.

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## Memory impairment associated with a dysfunction of the hippocampal cholinergic system induced by prenatal and neonatal exposures to bisphenol-A

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### Abstract

One of the most common chemicals that behaves as an endocrine disruptor is the compound 4,4'-isopropylidenediphenol, called bisphenol-A. In the previous study, we reported that exposure to bisphenol-A induced the abnormality of dopamine receptor functions in the mouse limbic area, resulting in a supersensitivity of drugs of abuse-induced pharmacological actions. The present study was undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnormalities such as anxiogenic behavior, motor learning behavior, or memory. In the present study, adult female mice were chronically treated with bisphenol-A-admixed powder food from mating to weaning. All experiments were performed using male pups. Here we found that prenatal and neonatal exposures to bisphenol-A failed to induce anxiogenic effects and motor-learning impairment using the light-dark test, elevated plus maze test, and rota-rod test. On the other hand, we found that prenatal and neonatal exposures to bisphenol-A induced the memory impairment using the step-through passive avoidance test. Immunohistochemical study showed the dramatic reduction in choline acetyltransferase-like immunoreactivity, which is a marker of acetylcholine (ACh) production, in the hippocampus of mice prenatally and neonatally exposed to bisphenol-A. These results suggest that chronic exposures to bisphenol-A could induce the memory impairment associated with the reduction in ACh production in the hippocampus.

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**Keywords:** Bisphenol-A; Memory; Choline acetyltransferase; Hippocampus; Endocrine disrupting chemicals

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. One of the most common endocrine disruptors is the compound 4,4'-isopropylidenediphenol, called bisphenol-A, which is used in the manufacture of many types of products.

Our recent studies suggest that exposure to bisphenol-A during prenatal and postnatal development has long-lasting effects on central dopaminergic systems linked to rewarding effects, as well as drug addiction induced by drugs of abuse [13–16,21,22]. The fetus uses natural hormonal messages that originate in its own hormone system and that of its mother for developmental guidance. Although bisphenol-A, has weak estrogenic activity,

prenatal and neonatal exposures to 17 $\beta$ -estradiol failed to induce the supersensitivity of morphine [13]. Furthermore, although it is well known that bisphenol-A disrupts thyroid hormone, prenatal and neonatal exposures to propylthiouracil, an inhibitor of thyroid hormone synthesis, reduced the activation of dopaminergic neurons (unpublished observation). These findings indicate that the disruption of dopaminergic neuron development induced by prenatal and neonatal exposures to bisphenol-A can be mediated by nonhormonergic actions of bisphenol-A. Furthermore, our preliminary biochemical studies showed that bisphenol-A has an affinity for nonspecific binding sites. Thus since its action site remained unclear, it is most likely that prenatal and neonatal exposures to bisphenol-A induce other behavioral abnormalities associated with the alternation not only of the dopaminergic system, but also of other neurotransmissions. The present study was then undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnor-

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malities such as anxiogenic behavior, motor learning behavior, and memory.

The low-dose endocrine disrupting actions of various environmental toxicants are serious problems. However, little is known about the effects on the central nervous system induced by low-dose bisphenol-A. We also investigated whether prenatal and neonatal exposures to low-dose bisphenol-A in mice could induce behavioral abnormalities.

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

All experiments were performed using 7–11-week-old male C57BL/6J mice (Japan SLC Inc., Shizuoka, Japan) that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd., Osaka, Japan). Adult female mice (10 weeks old) were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 30 ng/g diet (Low), and 2 mg/g diet (High) from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, the animals did not show any body weight loss. In the present study, at least 10 dams were used per group. We randomly selected a few pups per litter and housed to undergo the behavioral tests. To obtain unbiased results, we appropriately distributed mice for each behavioral study.

To investigate changes in anxiogenic-like effects, the mice were tested using the light–dark paradigm [2,19]. We used a box consisting of a small (18 cm × 13 cm × 18 cm) dimly lit compartment with black walls and a black floor, connected by a small opening (5 cm × 5 cm) to a large (18 cm × 18 cm × 18 cm) intensely lit (500 lux) compartment with white walls and a white floor. Each animal ( $n = 11–14$ /group, 7 weeks old) was placed in the dark compartment at the beginning of the observation session. The compartment entry and exit were defined as all four paws into and out of the chamber, respectively. The time spent in the lit compartment was recorded for 10 min. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

The elevated plus-maze consists of two opposing open arms (30 cm × 6 cm × 0.3 cm) and two opposing enclosed arms (30 cm × 6 cm × 15 cm) that are connected by a central platform (9 cm × 9 cm, 100 lux), thus forming the shape of a plus sign. Each animal ( $n = 5–7$ /group, 7 weeks old) was tested using the elevated plus-maze in each experiment. We recorded for 5 min the time spent in open or enclosed arms and the time spent entering into the arms. The results were calculated as mean ratios of the time spent in the open arms to the total time spent in both the open and enclosed arms. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

To investigate possible changes in motor learning impairment with prenatal and neonatal exposures to bisphenol-A, the mice were tested using the rota-rod performance procedure (rota-rod

test). The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a nonslippery surface. The 30-cm-long rod was placed at a height of 15 cm from the base and was divided into five equal sections by six disks. Thus the mice were tested simultaneously on the apparatus with a rod-rotating speed of 10 rpm. Each animal ( $n = 6–7$ /group, 9 weeks old) was placed on the rotating rod, requiring forward locomotion. Each animal was tested for a total of 5 min. If/when a mouse fell from the rod it was immediately replaced. We measured the time until falling the first time and the numbers of falls during the 5 min as the indicators of motor impairment twice a day. The score was the mean of latencies or numbers of falls in two trials. The data are presented as the mean with S.E.M. Statistical analyses were performed using two-way ANOVA with Bonferroni/Dunnett's test.

The experimental apparatus for the step-through passive avoidance test is a shuttle box that is divided into an illuminated small compartment (12 cm × 5 cm × 14 cm) and a dark/large compartment (25 cm × 25 cm × 20 cm) by a wall with a guillotine door. On the first day (conditioning day), each animal ( $n = 5–7$ /group, 11 weeks old) was placed in the illuminated compartment. After 90 s, the door was opened and the mouse was freely moved into the dark compartment. The door was closed as soon as the mouse stepped into the dark compartment, and an inescapable foot shock (0.5 mA, 0.5 s) was delivered through the grid floor. After 48 h from conditioning day, a retention test was started. The retention test was performed in a similar manner but without the electric shock and the step-through latency to the dark compartment was recorded. The maximal cut-off time for step-through latency was 30 min. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way or two-way ANOVA with Bonferroni/Dunnett's test.

In the immunohistochemical approach, the mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfusion fixed with 4% paraformaldehyde (pH 7.4). The brains were then quickly removed after perfusion, and thick coronal sections of the midbrain, including the hippocampus, were initially dissected using Brain Blocker. The brain coronal sections were postfixed in 4% paraformaldehyde for 2 h. After the brains were permeated with 20% sucrose for 1 day and 30% sucrose for 2 days, they were frozen in embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at  $-30^{\circ}\text{C}$  until use. Frozen 8- $\mu\text{m}$ -thick coronal sections were cut with a cryostat (CM1510; Leica, Heidelberg, Germany) and thaw-mounted on poly-L-lysine-coated glass slides. The brain sections were blocked in 3% normal horse serum with 0.2% triton (for choline acetyltransferase (ChAT); Chemicon International Inc., CA, USA) and 10% normal goat serum (NGS) (for NeuN; Chemicon) in 0.01 M PBS for 1 h at room temperature. The primary antibody of ChAT (1:100) or NeuN (1:250) was diluted in 0.01 M PBS containing 3% NHS with 0.2% triton or 10% NGS and incubated for 2 days at  $4^{\circ}\text{C}$ . The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 or Alexa 546 for 2 h at room temperature. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA). Fluorescence immunolabeling was detected using

a light microscope (AX-70; Olympus Optical, Tokyo, Japan) and photographed with a digital camera (Polaroid PDMCII/OL; Olympus Optical). Digitized images of the dentate gyrus were captured at a resolution of 140–200 pixels with a digital camera (Polaroid PDMCII/OL; Olympus). The density of ChAT labeling was measured with a computer-assisted imaging analysis system (Image J program, developed at the National Institutes of Health available at <http://rsb.info.nih.gov/ij>). The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity; this provided an image with immunoreactive material appearing in black pixels and non-immunoreactive material in white pixels. A standardized rectangle was positioned over the hippocampus of control mice. The area and density of pixels within the threshold value representing immunoreactivity were calculated and the integrated density was the product of the area and density. The same box was then “dragged” to the corresponding position on the hippocampus of bisphenol-A treated mice, and the integrated density of pixels within the same threshold was again calculated. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett’s test.

In the present study, prenatal and neonatal exposures to bisphenol-A failed to induce anxiogenic-like effects using the light–dark paradigm (Fig. 1A). As another measurement of anxiety, the mice prenatally and neonatally exposed to bisphenol-A were evaluated by the elevated plus-maze paradigm. The percentage of time spent in the open arms in the mice prenatally and neonatally exposed to bisphenol-A are shown in Fig. 1B. The prenatal and neonatal exposures to bisphenol-A failed to affect the percentage of time spent in the open arms. These results suggest that prenatal and neonatal exposures to bisphenol-A induced no anxiogenic-like behaviors. In the light–dark paradigm, the time spent in the light compartment of the mice prenatally and neonatally exposed to low-dose of bisphenol-A was increased as compared with the mice prenatally and neonatally exposed to high-dose of bisphenol-A (Fig. 1A). Although it is very difficult to explain the difference between the effect of low and high doses of bisphenol-A, it is necessary to consider the effects of the difference of the dosage on the difference of the action.

To investigate possible changes in motor learning impairment with prenatal and neonatal exposures to bisphenol-A, the mice were tested using the rota-rod test. In the present study, the mice prenatally and neonatally exposed to low or high doses of bisphenol-A progressively improved their skill in the rota-rod test, as the control mice did (Fig. 1C and D). The improvement of latency to fall and the number of fallings in the rota-rod test were indistinguishable between control and bisphenol-A treated mice (Fig. 1C and D). These results suggest that prenatal and neonatal exposures to bisphenol-A have no direct effect on motor skill learning.

The influence of prenatal and neonatal exposures to bisphenol-A on memory processes in mice was evaluated by the step-through passive avoidance test. In the conditioning trial, the step-through latency of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A was similar to that of control mice (Fig. 1E). Although the latency to step-

through increased in all groups compared to the latency shown at conditioning, the latencies to step into the dark compartment dramatically decreased in mice prenatally and neonatally exposed to bisphenol-A as compared to control mice ( $F_{(2,30)} = 5.766$ ,  $p < 0.05$ , Fig. 1F). These results strongly suggest that chronic treatment with low and high doses of bisphenol-A induced the memory impairment. The contextual fear conditioning is hippocampal-dependent memory. Therefore, we next investigated the morphological and/or functional changes in the hippocampus of mice prenatally and neonatally exposed to low and high doses of bisphenol-A.

Immunohistochemical study showed that prenatal and neonatal exposures to low and high doses of bisphenol-A dramatically decreased the level of choline acetyltransferase-like immunoreactivity (ChAT-IR) in the widespread regions of the hippocampus compared to control (Fig. 2Ai–iii). Especially as shown in high magnification images, cholinergic fiber was dramatically decreased in mice prenatally and neonatally exposed to low and high doses of bisphenol-A compared to control (Fig. 2Bi–iii). Furthermore, these phenomena were observed in several regions of the hippocampus, such as CA1, CA2, and CA3 (data not shown). Using semi-quantitative analysis, prenatal and neonatal low and high doses of bisphenol-A produced a significant decrease in the level of ChAT-IR in the hippocampus (Low:  $64.3 \pm 5.6\%$  of control mean, (\*\*\*)  $p < 0.001$  versus control mice; High:  $50.0 \pm 3.8\%$  of control mean, (\*\*\*)  $p < 0.001$  versus control mice, Fig. 2C). It is widely accepted that cholinergic function in the hippocampus is important for the learning and memory processes [1,4,12]. Among the cholinergic parameters described for the brains of Alzheimer’s disease patients, the decrease in ChAT activity is the most prominent and provides an excellent biochemical correlation to the severity of dementia. These reports strongly support our findings that the memory impairment corresponded well to the dysfunction of cholinergic neuron in the hippocampus of mice prenatally and neonatally exposed to bisphenol-A. Prenatal and neonatal exposure to bisphenol-A failed to affect the NeuN-IR in the dentate gyrus (Fig. 2Di–iii) and other regions of hippocampus compared to control, indicating that prenatal and neonatal exposure to bisphenol-A failed to cause cell death or layer formation on the mature pyramidal or granular cells in the hippocampus. Taken together, we hypothesized that although the prenatal and neonatal exposure to bisphenol-A could induce the dynamic morphological changes in cholinergic fibers associated with the disruption of the neuron development, it did not modify the hippocampal content of all immunogens.

It is widely accepted that one of the most common endocrine disruptors, bisphenol-A, has weak estrogenic activity. Moreover, endogenous estrogen,  $17\beta$ -estradiol, plays the critical role in the neurotransmission of the hippocampus associated with spinogenesis or neuroprotection [5,18]. Furthermore, many cholinergic neurons also express the growth-associated protein GAP-43, may be taken as a marker of neurite outgrowth [11]. Estrogens, among other factors, up-regulate the expression of GAP-43 in the developing and adult brain [9]. On the other hand, bisphenol-A also disrupts thyroid hormone. Thyroid hormone deficiency during brain development disrupts on the activities