Review Article

Bisphenol A: Developmental Toxicity from Early Prenatal Exposure

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Bisphenol A (BPA) exposure has been documented in pregnant women, but consequences for development are not yet widely studied in human populations. This review presents research on the consequences for offspring of BPA exposure during pregnancy. Extensive work in laboratory rodents has evaluated survival and growth of the conceptus, interference with embryonic programs of development, morphological sex differentiation, sex differentiation of the brain and behavior, immune responsiveness, and mechanism of action. Sensitive measures include RAR, aryl hydrocarbon receptor, and Hox A10 gene expression, anogenital distance, sex differentiation of affective and exploratory behavior, and immune hyperresponsiveness. Many BPA effects are reported at low doses (10–50 μg/kg d range) by the oral route of administration. At high doses (>500,000 μg/kg d) fetal viability is compromised. Much of the work has centered around the implications of the estrogenic actions of this agent. Some work related to thyroid mechanism of action has also been explored. BPA research has actively integrated current knowledge of developmental biology, concepts of endocrine disruption, and toxicological research to provide a basis for human health risk assessment. *Birth Defects Res (Part B)* 89:441–466, 2010. © 2010 Wiley-Liss, Inc.

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INTRODUCTION

This review includes two epidemiologic studies and numerous laboratory animal studies relevant to bisphenol A (BPA) developmental toxicity. There is also a large literature concerning BPA effects on development in wildlife, including aquatic species (Crain et al., 2007; European Union, 2008), which is not reviewed here. Also excluded from review were studies with exposures initiated after birth, studies with reports not available in English or without sufficient detail to evaluate, and studies made available after May 2009. BPA toxicity, including developmental toxicity, has previously been the subject of a number of reviews (CERHR, 2007; Richter et al., 2007; EU, 2008).

In this review, interpretation of results and discussion of relevance to human health, when presented, are generally based on the discussions of authors of the research reports that were reviewed.

We focus on studies with prenatal BPA exposure via maternal intake of BPA. Prenatal exposures in humans correspond to a more extended developmental period than in laboratory rodents, but prenatal rodent exposures can be appropriately generalized to human first and early second trimester development.

The review of animal studies is divided by endpoint:

- Embryo, fetal, and neonatal endpoints;
- Postnatal growth;
- Immune responsiveness;
- Effects on sex differentiation of the brain and behavior;
- Developmental neurobehavioral toxicity.

Most of the studies reviewed here used an oral route of exposure, either in food or drinking water, by gavage, or by oral installation (delivery of the dose into the mouth cavity). Injection routes were more common in studies of embryonic gene expression and fetal brain development (Tables 4 and 14). Because of extensive first pass metabolism, oral exposure would be expected to result in a lower internal dose than s.c. injection at the same mg/kg d administered dose (Edginton and Ritter, 2009).

The animal studies include routine toxicity studies conducted according to government agency guidelines as

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well as a larger number of research reports from the basic science literature. Basic research programs often use the same animal model (species, strain, route, and dose) in a series of hypothesis-testing studies. In this review, individual studies are presented in the context of other information from the same model. A similar approach was used in the European Union review of BPA (EU, 2008).

Because of BPA's potential estrogenic activity, comparison estrogens such as 17-β estradiol (E_2), ethinyl estradiol (EE), diethylstilbestrol (DES), methoxychlor (MXC), genistein, and nonylphenol are often used as a "positive control" in animal studies of BPA. Positive controls are useful in evaluating the sensitivity of the study design and endpoints. Although all these agents can be classified as "estrogenic," they differ in their effectiveness at the many different estrogen receptors and thus in their biological activity.

Developmental Toxicity Studies in Humans

Two studies of birth outcomes in humans have become available recently.

The stated objective of the first study (Padmanabhan et al., 2008) was to determine whether BPA is found in the circulation of pregnant women in the U.S. population. However, this study also examined the association between maternal plasma BPA concentrations and gestational length and birth weight. Blood samples from 40 mothers were collected at the time of delivery at the University of Michigan Hospital between August 2006 and November 2006. The authors did not specify how the mothers were selected to participate. The freely available form of BPA in plasma was measured using HPLC-MS/MS with a limit of detection (LOD) of 0.5 ng/ml. Maternal BPA concentrations (mean 5.9 ± 0.94 ng/ml, range 0.5–22.3 ng/ml) did not differ by maternal age, body mass index, or sex of the offspring. No differences were seen in length of gestation or birth weight relative to BPA concentrations ≤ 5 or > 5 ng/ml. Limitations identified in this study include the measures of BPA being obtained at birth and not earlier in gestation; the small sample size; the lack of control for potential confounders, such as race or socioeconomic status; and the lack of a measure of the glucuronide conjugates of BPA. Although the study did find levels of free BPA in maternal circulation, no measurements were made of BPA metabolites, BPA-glucuronide, and BPA-sulfate. Finally, an important limitation is that with the small sample size of only 40 mothers, the study lacked the power to detect significant effects on birth weight or length of gestation.

The second study (Wolff et al., 2008) used a prospective cohort to assess the association between prenatal exposures to phenols and phthalates and birth outcomes. Pregnant women were enrolled at Mount Sinai Hospital in New York City from March 1998 to March 2002. This ethnically diverse cohort included 404 primiparous women who had their first prenatal visit before 27 weeks gestation. A prenatal questionnaire was administered to the women during the third trimester to obtain information on pesticide and other environmental exposures, sociodemographics information, maternal health, and lifestyle habits. Maternal blood samples were taken and a urine sample was collected mostly during the third trimester. Birth outcome information was acquired from the hospital database. A concern for the study in general is the number of multiple comparisons conducted in the analyses (72), which could have led to spurious significant associations. Urine samples were analyzed for 5 phenols and 10 phthalate urinary metabolites. BPA was analyzed using HPLC-MS (LOD = 0.4 μg/l). Sufficient specimen amounts were available for phenol analysis in 367 women. Of the phenols, BPA, 2,5-dichlorophenol (2,5-DCP), and 2,4-dichlorophenol were positively correlated with maternal prepregnancy body mass index if the biomarker was expressed as micrograms per liter, but not if expressed as micrograms per gram creatinine. None of the phenols were significantly associated with any birth outcomes in regression models, adjusted for covariates. Birth outcomes examined in this study included birth weight, birth length, head circumference, and gestational age. Interaction terms revealed possible sex-specific effects in four models of birth weight or birth length for three phenols (2,5-DCP, triclosan, and benzophenone-3). No effects were observed with BPA; however, as noted by the authors, urinary concentrations of BPA were much lower than those of 2,5-DCP, triclosan, and benzophenone-3. As shown below (Table 1), BPA levels in these women were considerably lower than the levels reported in women from the National Health And Nutrition Examination Survey (NHANES), while levels of triclosan and 2,5-DCP were higher. At the upper end of the distributions, levels of triclosan, benzophenone-3, and 2,5-DCP, but not BPA, were much higher in these women than the levels reported for women in NHANES. The exposure assessment for BPA relied on a one-time measure during the third trimester. Since the half-life of BPA is short, this may not be a reliable indicator of exposure throughout or during critical periods of pregnancy.

Low BPA urine concentrations were reported in pregnant women by Wolff et al. (2008) relative to population norms, but other studies have found similar or higher urine concentrations in comparison to various reference populations (Vandenberg et al., 2010). When pregnant and nonpregnant women were compared in the same study slightly higher concentrations were associated with pregnancy. Serum BPA concentrations were higher than population norms in several studies, but two studies directly comparing pregnant and nonpregnant women found similar or slightly lower concentrations (Vandenberg et al., 2010). Comparison of biomonitoring studies is hindered by differences in the number of samples obtained per subject and the timing of sampling relative to delivery.

Developmental Toxicity Study in a Nonhuman Primate

A single, recent study in nonhuman primates (cynomolgous monkeys) is available (Nakagami et al., 2009). Pregnant monkeys ($n = 18$) were exposed to BPA via an implant that released 10 μg/kg d, equivalent to an oral dose of 5 mg/kg d to rats based on the investigator's previous pharmacokinetic studies (Negishi et al., 2004b; Tominaga et al., 2006). The exposure continued from gestation day (GD) 20 to birth ($\sim$GD 160). No differences from controls ($n = 19$) were found in fetal loss, stillbirth, neonatal death, gestation length, or birth weight. After birth, videotapes were made of mother–infant pairs at

Table 1
Urinary Levels of BPA and Other Phenols in Women as Reported by NHANES and by Wolff et al. (2008)

	Geometric mean (95% CI) (μg/l)	50th percentile	75th percentile	95th percentile (NHANES) maximum (Wolff et al.)
BPA				
NHANES	2.4 (2.1–2.8)	2.4	5.0	20.1
Wolff et al.		1.3	2.3	35.2
Triclosan				
NHANES	10.6 (9.3–12.1)	7.4	33.2	430
Wolff et al.		11	42	1,790
Benzophenone-3				
NHANES	30.7 (23.7–39.8)	26	137	1,790
Wolff et al.		7.5	31	92,700
2,5-DCP				
NHANES	[a]	1.41	24.6	1,320
Wolff et al.		53	135	13,300

BPA, bisphenol A; NHANES, National Health And Nutrition Examination Survey; 2,5 DCP, 2,5-dichlorophenol.
[a]Not calculated. Proportion of results below the limit of detection was too high to provide a valid result. NHANES values: 2,5-DCP values were from the third report, survey years 2001–2002 (CDC, 2005); BPA (Calafat et al., 2008c), triclosan (Calafat et al., 2008b), and BP3 (Calafat et al., 2008a) were from survey years 2003–2004.

intervals prior to weaning and extensive statistical analysis was conducted of behaviors coded in 14 categories (for infants and mothers) from the videotapes. BPA-exposed males and their mothers showed differences from respective controls. For the infants, behaviors affected were clinging, outward looking, and social exploration; for the mothers, outward looking was affected. Multivariate analysis of these behaviors and two others (proximity and environmental exploration) generated principal component scores for each infant. BPA-exposed male infants and their mothers showed statistically significant differences from controls for a composite score based on the principal components scores. This analysis was interpreted as supporting a difference in sexual differentiation of behavior due to BPA exposure in the male infants and their mothers.

DEVELOPMENTAL TOXICITY STUDIES IN LABORATORY RODENTS

Embryo and Fetal Endpoints and Pregnancy Outcome

Regulatory guideline studies using rodents. The U.S. Environmental Protection Agency (EPA), the European Union (EU), and other governmental agencies have established guidelines for routine testing for developmental and reproductive toxicity in laboratory animals. These studies provide toxicity endpoints in both mother and fetus/offspring. Routine guideline-type studies are outlined in Table 2. In this table, as well as subsequent tables in this review, a reference to other review documents with more complete descriptions of each study is provided.

There are two oral developmental toxicity studies (Morrissey et al., 1987; Kim et al., 2001), one study with several subdivisions using the Reproductive Assessment by Continuous Breeding (RACB) protocol (Morrissey et al., 1989), two rat multigeneration studies (Ema et al., 2001; Tyl et al., 2002b), and one mouse multigeneration study (Tyl et al., 2007).

One of the three available developmental toxicity studies is a small study with intraperitoneal (i.p.) administration of BPA to rats. This study was sponsored by the National Institute of Occupational Safety and Health (NIOSH) and briefly reported in a paper that included studies of 18 other chemicals (Hardin et al., 1981). Number of live fetuses per litter and fetal body weight and length were adversely affected at both doses used. Maternal toxicity was not reported. Morrissey et al. (1987) reported more comprehensive studies in both mice and rats with gavage administration during embryogenesis. No developmental or maternal toxicity was reported in the rat study. The mouse study reported a significant linear trend for increased resorption and decreased fetal body weight as well as a significant difference from controls at the highest dose (1250 mg/kg d). This dose also caused 18% mortality in the dams. A more recent rat study with gavage administration (Kim et al., 2001) used a more extended BPA administration from GD 1 to GD 20 and also a wider dose range than the Morrissey et al. study. This study found BPA effects on pregnancy completion ratio, pre- and post- implantation loss, number of live fetuses/litter, fetal weights, and delayed ossification, with most effects occurring at the highest dose tested (1000 mg/kg d) and no significant effects at the lowest dose (100 mg/kg d). Among the measures of maternal toxicity, maternal weight, weight gain, and corrected body weight gain (weight gain minus uterine contents) were affected at the two higher doses. There was no maternal mortality. There are no developmental toxicity studies conducted according to current U.S. EPA guidelines.

Although no comparison estrogens were used in these guideline developmental toxicity studies, effects on fetal viability and weight are consistent with hypothesis-testing studies of the effects of estradiol on pregnancy in the rat (Bartholomeusz et al., 1999; Matsuura et al., 2004). For instance, Matsuura et al. administered E_2 (as estradiol benzoate) to Wistar rats on GD 12–19 and found a dose-dependent reduction in fetal survival and fetal weight on GD 20. Smaller placentas, degeneration of trophoblasts, and reduction of fetal vessels in the placental labyrinth were also observed after the estradiol treatment, thus implicating placental mediation of fetal effects. The lowest effective dose (10 μg E_2/day by injection) produced a nonsignificant 47% increase in maternal plasma estradiol.

Table 2
Routine Guideline-Type Studies With Data on Pregnancy Outcome After Prenatal BPA Exposure

Study	Design	General toxicity	Pregnancy outcome
Female exposure (*developmental toxicity studies*)			
Hardin et al. (1981)	SD rats	25% preg/inseminated,	↓ live fetuses per litter
EU (2003)	GD 1–15	125 mg/kg d	↓ fetal body weight
	i.p.	100% pregnancy completion	↓ fetal body length
	0, 85, and 125 mg/kg d	$N = 4$	Both doses
Morrissey et al. (1987)	CD-1 mice	Dam mortality 18%,	↑ % resorptions/litter,
	GD 6–15	1250 mg/kg d	1250 mg/kg d
NTP-A	gavage	corrected maternal weight gain	↓ fetal body weight,
EU (2003)	0, 500, 750, 1000, and 1250 mg/kg d	71% of control, 1250 mg/kg d	1250 mg/kg d
	$N = 21–26$		Linear trend tests significant
	CD rats	No dam mortality 640 corrected	No effects
	GD 6–15	maternal weight gain 66% of	
	gavage	control	
	0, 160, 320, and 640 mg/kg d		
	$N = 25–29$/group		
Kim et al. (2001)	SD rats	↓ dam body weight	↓ live pups/litter
NTP-A	GD 1–20	↓ dam body weight gain	↓ male pup weight/litter
	0, 100, 300, and 1000 mg/kg d,	↓ corrected dam body weight	↓ female pup weight/litter
	gavage	gain	↓ skeletal ossification
	$N = 14–20$		
Male and/or female exposure (*RACB studies*)			
Morrissey et al. (1989)	Swiss mice	↓ dam weight at delivery,	↓ litters/pair,
	14 wk, begin 1 wk before mating	↑ days to litter	↓live pups/pair,
NTP-A	Diet		↓ live males/females/litter,
EU (2003) (cited as NTP, 1985b)	0, 0.25, 0.5, and 1% diet		% pups born alive
	470, 900, and 1880 mg/kg d		↑ live pup weight/litter
	Male and female exposure		↑ live male pup weight/litter
			↑ live female pup weight/litter
Morrissey et al. (1989)	Swiss mice	↓dam weight; ↑dam adjusted	↓ live pups/litter
	14 weeks, begin 1 week before	liver and kidney weights	↓ live male pups/litter
NTP-A	mating		↓ live female pups/litter
EU (2003) (cited as NTP, 1985b)	Diet		↑ live male pup weight/litter
	1920 mg/kg-d		↑ live female pup weight/litter adjusted
	Female exposure only		
Male and female exposure (*multigeneration studies*)			
Ema et al. (2001)	SD rats Two-generation Gavage	No mortality or effects on dam	No effects: pups/litter, sex ratio;
NTP-A	0, 0.2, 2, 20, and 200 μg/kg d	weight;	pup weight PND 0
EU (2008)	$N = 19–24$	No weight gain data presented	
Tyl et al. (2002b)	SD rats	↓ dam body weights and	↓ live pups/litter
NTP-A	Three-generation	weight gain gestation and	↓ pups/litter
(cited as Tyl et al., 2000a,b, 2002b)	Diet	lactation	↓ implantation sites
	0, 0.001, 0.02, 0.3, 5, 50, and		Comparison estrogen, estradiol 2.5 mg/kg
EU (2003)	500 mg/kg d		No effect M,F body weight
	Male and female exposure		Trend across doses live pups; no trend analysis; no weight trend
			↓ live pups/litter
			↓ pups/litter
			↓ implantation sites
Tyl (2008), Tyl et al. (2008a,b)	CD-1 mice	No effects on dam weight or	Significant ANOVA; No
	Two-generation	dam weight gain; ↑ rel liver	pairwise effects
NTP-A	Diet	weight; ↑ kidney weight	↓ live pups/litter
(as Tyl et al., 2006)	0, 0.003, 0.03, 0.3,5, 50, and		↓ total pups/litter
EU (2008)	600 mg/kg d		↓ stillbirth index
	$N = 55$ (control), 19–25 (BPA)		
Tyl et al. (2002a)	CD-1 mice,	↓ dam body weights and	↓ live pups/litter
NTP-A (cited as Tyl et al., 2002a)	1-generation	pregnancy weight gain; ↑	↓ total pups/litter
	0, 437, 875, and 1750 mg/kg d	liver kidney weights, ↑	Significant dose trend ↓ female
	during gestation	gestation length	PND0 bw
Tyl et al. (2006)	CD-1 mice	No effects on maternal weight,	↓ live pups/litter
EU (2008) (as abstract)	Two-generation	maternal weight gain, liver	↓ total pups/litter
	0, 0.001, 0.005, 0.05, and 0.5 ppm E_2	weight or kidney weight	↑ pup weight
(E_2 study)	0, 0.2, 1.0, 1, 30, and 100 mg E_2/kg d		

Table 2
Continued

Study	Design	General toxicity	Pregnancy outcome
Other studies with pregnancy outcome data			
Tinwell et al. (2002) NTP-A EU (2008)	SD, Wistar rats GD 6–21 20, 100 μg/kg d, 50 mg/kg d, gavage $N = 7$/group	No effects on maternal weight	No effects litter size, sex ratio, birth weight
Negishi et al. (2003) NTP-A EU (2008)	F344 rats 0, 4, 40, and 400 mg/kg d GD 10–PND 20 Gavage $N = 8$, 9/group	40, 400 ↓ maternal body weight during pregnancy	No effect: total pups/litter, sex ratio at birth; no birthweight measure

NTP-A: full-study description in the NTP-CERHR Expert Panel Report within the "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A," September 2008. NTP-B: full-study description in the NTP Brief within the "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A," September 2008. EU 2003: full-study description in "European Union Risk Assessment Report: 4,4′-isopropylidenediphenol (bisphenol-A)," 2003. EU (2008): full-study description in "Updated European Risk Assessment Report 4,4′-Isopropylidenediphenol (bisphenol-A)," April 2008. SD, Sprague–Dawley; BPA, bisphenol A; PND, postnatal day; GD, gestation day.

In addition to the developmental toxicity studies, a larger number of recent routine guideline reproductive toxicity studies are available for BPA, which contain measures such as litter size, pup weight, and mortality. These pregnancy outcome variables may reflect prenatal developmental toxicity in the F_0 generation (dams exposed to BPA before and during pregnancy only). Pregnancy outcome in later generations may be influenced by effects on reproductive tract maturation in the breeders.

Some of these routine guideline studies include a comparison estrogen as a positive control. Separate studies of estrogenic agents using these study designs are also available for comparison (Biegel et al., 1998a,b; Tyl et al., 2008b).

Multigeneration studies of BPA in both rats and mice have been published in the peer-reviewed literature (Tyl et al., 2002b, 2008a) and were also made available for this review as the original study reports (Tyl et al., 2000, 2007). Additional work relevant to this review include a one-generation BPA study in mice, a two generation study of E_2 in mice, and a 13-week BPA dose range-finding study in mice (Tyl et al., 2002a, 2005, 2006). Details of these studies are presented in Table 2.

In terms of pregnancy outcome in the first generation, the multigeneration studies were consistent in finding BPA and E_2 effects on pup survival. The BPA study in rats (Tyl et al., 2000) found treatment effects by analysis of variance (ANOVA) on two indices related to pup survival:

- Total number of pups on the day of birth, postnatal day (PND) 0.
- Number of live pups on PND 0.

There was also a significant linear trend across doses for these parameters, and a significant difference from control in the high dose group for both measures in the rat study. The BPA study in mice (Tyl et al., 2007) also found significant treatment effects by ANOVA for a number of variables reflecting pup survival:

- Number of live pups on PND 0.
- Total number of pups on PND 0.
- Number of dead pups on PND 0.
- Stillbirth index on PND 0.
- Live birth index on PND 0.

No dose trend analysis was presented. None of the pairwise comparisons of a BPA-treated group with the combined control group was significant in the mouse study using the statistical approaches of the study. These pup survival measures in mice were also affected by the comparison estrogen E_2 at a dose of 0.5 mg/kg d (Tyl et al., 2007).

There were no effects on pups' birth weight in either the rat or the mouse study. However, it is important to note that BPA led to increased gestation length in the mouse study and calculations of birth weight were not corrected for gestational age, which can influence birthweight. Also pup weights were not corrected for litter size, which was influenced by BPA. Pup weights in the high dose group began to fall behind after birth and were significantly lower than controls by PND 7. In the rat study, there was no effect on pup birth weights but a significant treatment effect and a linear dose trend were seen for pup body weights by PND 4. As was the case in the mouse study, pup birthweights were not corrected for gestation age or litter size.

Although there was no comparison estrogen in the rat three-generation study, pregnancy outcome in the first generation was very similar to that in an estradiol one-generation study (Biegel et al., 1998b) in finding decreased pups/litter, live pups per litter, and implantation sites. Using litter size and sex ratio as covariates in the analysis, the Biegel study also found an effect on birthweight. Gestation length was not reported.

In addition to these multigeneration studies, Ema et al. (2001) conducted a multigeneration study in rats based on Japanese and international guidelines, and characterized by gavage administration of BPA and a low dose range ($\leq$0.2 mg/kg d). This study found no effects on litter size or pup birth weight in the first generation.

Another type of routine guideline study is the Reproductive Assessment by Continuous Breeding (RACB), in which dosing begins shortly before mating in both parents and continues through the birth of

several litters over a 90-day period. There are three relevant RACB guideline studies using BPA and reported together (Morrissey et al., 1989) (Table 2):

- A study using subcutaneous (s.c.) administration via minipump to the dam.
- A study using dietary exposure of both parents.
- A study using dietary exposure of the dam only.

Notably, the implanted minipump study reported *increased* litter sizes and pup birth weights in the BPA-exposed groups. However, the study report cited problems with retention of the implants, which led the investigators to disregard the data. The diet studies, with either both parents or dam only exposed, reported decreased litter sizes but increased pup birth weights. It is possible that the greater birth weights were secondary to the smaller litter sizes in the BPA group. Corrections for litter size were not performed in the statistical analysis. BPA effects in the RACB studies occurred at doses >900 mg/kg-d. Minimal maternal/paternal toxicity data were included in the study report.

In order to provide information on maternal/paternal toxicity at the dose levels used in the RACB studies, an "abbreviated" one-generation study was conducted (Tyl et al., 2002a) using the effective doses from the RACB study and the same mouse model (CD-1). In this design, only one litter was produced by each mating pair and the duration of exposure prior to mating was two weeks (rather than 10 weeks in the multigeneration studies). The study identified maternal/paternal toxicity as increased liver and kidney weights and pathology (reflected in histopathology and clinical chemistry values). Reproductive toxicity included prolonged gestation length and decreased litter size (total and live pups per litter). Increased liver and kidney weights in the parents, first identified in the one-generation study, were seen in the later multigeneration study of BPA.

In general these studies suggest that fetal viability is sensitive to BPA at doses >500 mg/kg d by the oral route in these experimental paradigms. Based on limited data, malformations and variations do not seem affected by BPA, but studies conducted according to current guidelines are not available. Fetal/newborn weights were variably affected but evaluation of this endpoint is complicated by BPA-induced increase in gestation length and decrease in litter size, both of which would tend to increase fetal/newborn weights.

Table 3 demonstrates the varying statistical approaches to the evaluation of key pregnancy outcome variables in the routine guideline-style multigeneration studies. Different tests used (ANOVA and trend test), as well as different statistical programs and approaches to data summary, prevent a simple agree/disagree conclusion when comparing two studies. As is the case throughout the BPA literature, findings must be individually examined and integrated.

In addition to the choice of statistical tests, the recent multigeneration studies (Tyl et al., 2000, 2007) differ from other studies reviewed here in several aspects of the approach to statistical analysis. In these studies "statistical outliers" were eliminated from the data sets of some variables prior to analysis and not included in the tables of individual animal data. The criteria for the determination of "statistical outlier" and the reasons that some variables, but not others, were selected were not stated. In addition "unrealistic" values were excluded from the data analysis and reports of individual data. The statistical analysis also used one-sided rather than two-sided group mean comparisons for some variables. Also, the statistical analysis used "robust regression" analyses when homogeneity of variance assumptions were not met for ANOVA (Levene's test). Other studies reviewed here used nonparametric statistical analyses under these conditions. Although the studies were fairly consistent in their statistical approach, dose trend tests were included for some studies (mouse developmental toxicity, rat three-generation, mouse one-generation) but not others (mouse BPA and E_2 two-generation). Finally, sex differences were not evaluated statistically for any endpoint in these studies. Rather, data from the two sexes were evaluated separately or pooled.

Basic research studies. Many studies of BPA and embryo/fetal development have focused on possible estrogenic effects at the early stages of development. Independent studies have documented the distribution of BPA and BPA metabolites from mother to conceptus during pregnancy (Miyakoda et al., 1999, 2000; Domoradzki et al., 2003; Zalko et al., 2003; Kawamoto et al., 2005). They note that BPA, but not BPA glucuronide, distributes readily to the fetus.

Because estrogen receptors are expressed at various stages of embryo/fetal development both prior to and after differentiation of the fetal gonads and fetal production of steroid hormones, there are many potential sites of action for exogenous estrogen during in utero development.

Studies of the effects of exogenous estrogen on different stages of embryonic development are not available in laboratory rodents. However, this topic has been recently studied in some detail in zebrafish (Brion et al., 2004; Fenske et al., 2005; Schafers et al., 2007; Soares et al., 2009).

Preimplantation effects: Estrogen receptor mRNA is detected in the early 2-cell embryo as well as in the blastocyst (Hou and Gorski, 1993; Hiroi et al., 1999). To determine the potential impact of BPA on preimplantation embryos, 2-cell mouse embryos were cultured in medium containing BPA with or without the estrogen receptor blocking agent tamoxifen (Takai et al., 2000). Lower concentrations (1 and 3 nM BPA) accelerated embryonic development, increasing the percentage of 2-cell embryos advancing to blastocysts after 48 hr in culture. At a higher concentration (100 μM) blastocyst formation was suppressed. Tamoxifen blocked both these effects, indicating that they could be estrogen-receptor mediated. Blastocyst morphology and cell counts did not differ from controls (Takai et al., 2001). When BPA-exposed blastocysts were transferred to the uterus of pseudopregnant mice, pregnancy and birth parameters did not differ from control, but offspring were heavier at weaning (Takai et al., 2001) (see Table 5). Acceleration of embryonic development at low doses of BPA has also been shown in a fish model (Ramakrishnan and Wayne, 2008).

Gene expression in the embryo/fetus: Some studies used "upstream" indicators such as gene expression and proteomics to demonstrate biological actions of BPA relevant to in utero development (Table 4). Some of these studies used an injection of BPA into the pregnant dam,

Table 3
Statistical Approaches Used to Evaluate the Same Endpoints in Different in Guideline Studies of BPA and E_2

Reference type of study	Implantation #	Pups/litter	Live pups/litter	Fetal weight
Morrisey et al. (1987) BPA rat dev tox	ANOVA/Dunnett's or Williams post hoc, one-tailed dose trend test	Measure not reported	ANOVA/Dunnett's or Williams post hoc, one-tailed dose trend test	ANOVA/Dunnett's or Williams post hoc, two-tailed, dose trend test; not adjusted
Kim (2001) BPA rat dev tox	Kruskal–Wallis ANOVA/Mann–Whitney post hoc tests	Measure not reported	ANOVA/post hoc Scheffe test	ANOVA/post hoc Scheffe test; not adjusted
Biegel et al. (1998a,b) E_2[a] E_2 rat multigen	Dose trend test (Jonckheere)	Dose trend test (Jonckheere)	Dose trend test (Jonckheere)	Dose trend test (Jonckheere) adjusted for litter size, sex ratio
Tyl et al. (2002b) BPA rat multigen	ANOVA/Dunnett's post hoc test one-tailed dose trend test	ANOVA/Dunnett's post hoc test, one-tailed dose trend test	ANOVA/Dunnett's post hoc test, one-tailed dose trend test	ANOVA/Dunnett's post hoc test, two-tailed dose trend test; not adjusted
Morrisey (1987) BPA mouse dev tox	ANOVA/Dunnett's or Williams post hoc, one-tailed linear trend test	Measure not reported	ANOVA/Dunnett's or Williams post hoc, one-tailed linear trend test	ANOVA/Dunnett's or Williams post hoc, two-tailed linear trend test; not adjusted
Tyl et al. (2008b) E_2 mouse multigen	ANOVA/Dunnett's post hoc test, one-tailed	ANOVA/Dunnett's post hoc test, one-tailed	ANOVA/Dunnett's post hoc test, one-tailed	ANOVA/Dunnett's post hoc test, two-tailed; not adjusted
Tyl et al. (2002a) BPA mouse one gen	ANOVA/Dunnett's post hoc test, one-tailed dose trend test	ANOVA/Dunnett's post hoc test, one-tailed dose trend test	ANOVA/Dunnett's post hoc test, one-tailed dose trend test	ANOVA/Dunnett's post hoc test, two-tailed dose trend test; not adjusted
Tyl et al. (2008a) BPA mouse multigen	ANOVA/Dunnett's post hoc test, one-tailed	ANOVA/Dunnett's post hoc test, one-tailed	ANOVA/Dunnett's post hoc test, one-tailed	ANOVA/Dunnett's post hoc test, two-tailed; not adjusted
Ema et al. (2001) rat multigen	ANOVA/Dunnett's post hoc tests one-/two-tailed; not stated	ANOVA/Dunnett's post hoc tests one-/two-tailed; not stated	ANOVA/Dunnett's post hoc tests one-/two-tailed; not stated	ANOVA/Dunnett's post hoc tests; not adjusted

ANOVA, analysis of variance; BPA, bisphenol A.
[a]ANOVA was not mentioned in the statistical procedures for these endpoints in this study.

either by the subcutaneous route, which avoids first pass metabolism, or by the i.p. route, which includes first pass metabolism. Thus, dose-related effects are difficult to compare across studies.

Estrogen receptor activation by BPA in utero was demonstrated in GD 13.5 transgenic mouse fetuses carrying a luciferase reporter gene with an estrogen response element (Lemmen et al., 2004). DES was also active in this assay at similar concentrations to BPA. Using an in vitro system with the luciferase reporter, BPA had 3–4 orders of magnitude lower potency than DES, suggesting that fetal estrogenic sensitivity in utero is greater than indicated by isolated cell assays.

Other studies compared gene activation patterns of BPA to those of 17α-EE and genistein in the reproductive organs of GD 20 rat fetuses (Naciff et al., 2002, 2005). The BPA exposure was via s.c. injection of the dam on GD 11–20 and fetuses were collected 2 hr after the last dose. For the ovary and uterus, EE and BPA had similar expression profiles as reflected in microarray data and confirmed by PCR, but only at the higher doses (400 mg BPA/kg vs. 10 μg EE/kg) (Naciff et al., 2002). In testis/epididymis, higher doses of all three agents (BPA 400 mg/kg d, EE 10 μg/kg d, and genistein 100 mg/kg d) showed common activation of 50 genes (Naciff et al., 2005). These studies indicate that BPA can activate estrogen-mediated gene transcription in the embryo/fetus. At doses greater than 1 mg/kg d by injection, the BPA effects were weaker than those of the comparison estrogen. The injection route avoids first pass glucuronidation, providing a better comparison between the parent compounds.

Links between altered gene expression in fetal reproductive organs and abnormal development of these organs have not yet been made. However, BPA effects on fetal chromosomes have been shown to carry over to the pubertal ovary. In a study of fetal ovary (Susiarjo et al., 2007), chromosome abnormalities were detected in oocytes of GD 18 mouse fetuses who had been exposed to 20 μg/kg d BPA from 11.5 days post conception (dpc). Specifically, oocytes entering meiosis had abnormal chromosome alignment and a greater frequency of recombination. When oocytes were examined postnatally at 4–5 weeks of age, higher rates of aneuploidy were found in prenatally BPA exposed mice (21.4%) when compared to controls (1.8%). A comparison estrogen was not used in this study, but the authors suggest an anti-estrogenic mechanism based on the finding of similar oocyte abnormalities in estrogen receptor β knock out

Table 4
Effects of BPA on Gene Expression in the Embryo/Fetus

Reference	Species Route	Dose—Time of exposure	Endpoints—Time of assessment	Findings
Nishizawa et al. (2003) NTP-A EU (2008)	ICR mice oral	2 μg/kg d GD 6.5–17.5	RARα RXRα mRNA Cerebrum, cerebellum, Gonads, Liver 12.5, 14.5 18.5 dpc	General decrease in RAR and RXR expression, age- and sex-dependent ↑ RARα cerebella, 12.5 dpc No comparison estrogen
Nishizawa et al. (2005a) NTP-A	ICR mice oral	0, 0.02, 2, 200, and 20,000 μg/kg d GD 6.5–13.5 GD 6.5–17.5	AhR, RAR,RXR mRNA 14.5, 18.5 dpc	↑ AhR, RAR, RXR mRNA, brain, and gonads, depending on does, sex, and age No comparison estrogen
Nishizawa et al. (2005b) NTP-A	ICR mice oral	0, 0.02, 2, 200, and 20,000 μg/kg d GD 6.5–13.5 GD 6.5–17.5	AhR ARNT AhRR GST Cyp1A1	General increase in expression, dose and age dependent; Comparison estrogen E_2
Imanishi et al. (2003) NTP-A EU (2008)	ICR mice oral	2 μg/kg d GD 6.5–17.5	Placenta; microarray of 20 nuclear receptor mRNA dpc 18.5	Sex dependent pattern of activation/repression No comparison estrogen
Lemmen et al. (2004) NTP-A	Transgenic mice; C57BI/6J × CBA F_1 injection (i.p.)	13.5 days post conception (dpc) 10–10,000 μg/kg	Luciferase with ERE 8, 24 hr after treatment	ERE activation at 1000 and 10,000 μg/kg d in embryos 8 hr after dosing; Comparison estrogens E_2 propionate, DES
Susiarjo et al. (2007) NTP-A EU (2008)	Pregnant C57BL/6 mice implanted time-release BPA pellets ERα KO mice	GD 11.5 (for 1 week) 20 μg/kg d (pellets released 400 ng BPA daily)	Chromosome analysis of oocytes of GD 18 fetuses	Chromosome abnormalities in oocytes during meiosis 20 μg/kg d No comparison estrogen
Naciff et al. (2002) NTP-A EU (2008)	SD rats injection (s.c.)	GD 11–20 0, 5, 50, or 400 mg/kg d (1 ml/kg bw of dose solution, controls received DMSO)	Gene activation in ovaries and uterus of GD 20 fetuses	Gene activation pattern similar to EE 400 mg/kg d Comparison estrogens genistein and EE
Naciff et al. (2005) NTP-B EU (2008)	SD rats injection (s.c.)	GD 11–20 0, 5, 50, or 400 mg/kg d (1 ml/kg bw of dose solution, controls received DMSO)	Gene activation in testes and epididymis of GD 20 fetuses	50 genes showed common activation for all three agents Comparison estrogens genistein and EE
Smith and Taylor (2007)	CD-1 mice injection (i.p.)	0.5, 1.0,5.0, 50, and 200 mg/kg d GD 9–16	Uterine stromal cell HOXA10 expression 2–6 wk postnatal	↑ HOXA10
Moriyama et al. (2002) NTP-B EU (2008)	In vitro studies	100 μM–1 nM	TR binding and activation	↓ T_3 binding to TR ↓ TR activation of gene transcription
Yaoi et al. (2008)	ICR/Jc mice injection (s.c.)	20 μg/kg d GD 0–12.5 or 14.5	Mouse embryo forebrain methylation assay with follow-up cloning	↑ and ↓ methylation target sites in CGI and promoter regions of genes relevant to brain development No comparison estrogen
Dolinoy et al. (2007) NTP-B	Mice a/a (Avy/a embryos)	50 mg/kg diet 2 weeks premating, pregnancy, lactation	Offspring coat color, methylation at relevant sites of the Agouti gene promoter	Shift in distribution of offspring coat color; 30% hypomethylation; reversible by feeding methyl donors

BPA, bisphenol A; EE, ethinyl estradiol; GD, gestation day.

(ERβ (−/−)) mice. ERα (−/−) mice did not show these chromosome abnormalities in oocytes.

Embryonic, fetal, and placental gene expression after BPA exposure were studied in experiments that focused on retinoic acid (RA) receptors (RAR and RXR) and aryl hydrocarbon receptors (AhR). RA acts by binding to RA receptors RAR and RXR, which dimerize to form transcription factors. Metabolism of retinol (vitamin A) to RA involves enzymes regulated by the AhR receptor. AhR is also involved in degradation of the estrogen

Table 5
Studies Reporting Changes in Offspring Weight After In Utero Bisphenol a With or Without Postnatal Exposure

Reference	Species	Route	Time	Dose	Findings
Low-dose studies					
Takai et al. (2001) NTP-A EU (2003)	B6C3F1 mouse embryos ICR mouse dams	Embryo culture	GD 1–3	1 nM 100 μM	↑ body weight PND 21, both doses
Howdeshell et al. (1999) **Howdeshell and vom Saal (2000)** NTP-A	CF-1 mouse	Oral (dam)	GD 11–17	2.4 μg/kg d	↑ body weight PND 21 in females positioned next to one or two other females in utero
Rubin et al. (2001) NTP-A EU (2003)	SD rats	Drinking water	GD 6 through lactation	0.1 and 1.2 mg/kg d	↑ body weight PND 4, 7, 11 males and females, both doses; ↑ body weight PND 28–110 females low dose
Miyawaki et al. (2007) NTP-B	ICR mice	Drinking water	GD 10–PND 30	0.3 and 3 mg/kg d	↑ body weight PND 31, females both doses, males high dose
High-dose studies					
Negishi et al. (2003) NTP-A EU (2008)	F344 rats	Oral	GD 10–PND 20	0, 4, 40, and 400 mg/kg d	↓ body weight 40, 400 mg/kg d, PND 7, 21, 28 (PND 56 males only 400 mg/kg d)
Takagi et al. (2004) NTP-A EU (2008)	SD rats	Diet	GD 15–PND 10	0, 60, 600, and 3000 ppm 0, 5, 49, and 232 mg/kg d	↓ body weight gain neonates, PND 2–10, 232 mg/kg d
Matsumoto et al. (2004) NTP-A	ddY mice	Diet	GD 14–PND 7	0, 10,000 ppm 0, 1000 mg/kg d	↓ body weight PND 1–7

SD, Sprague–Dawley; PND, postnatal day; GD, gestation day.

receptor ERα. In cell culture studies, BPA has been shown to both increase and decrease AhR gene transactivation depending on dose (Bonefeld-Jorgensen et al., 2007).

In a series of papers, Nishizawa et al. advanced the hypothesis that BPA can activate AhR expression in embryos, thus subsequently altering RA action during embryogenesis. BPA was administered orally during organogenesis to mice and embryos were collected for the analysis of mRNA expression by RT-PCR. Specifically, the brain (cerebrum and cerebellum) and gonads (ovaries and testes) were assessed on GD 12.5, 14.5, and 18.5 embryos 24 hr after discontinuation of treatment begun on GD 6.5. Across the dose range examined (0.02–20,000 μg/kg d), BPA generally increased AhR expression in brain (cerebrum and cerebellum) and gonads of the embryos, an effect similar to the positive control E_2 (5 μg/kg d). In further support of AhR activation, expression of two other genes in this pathway was also increased. CYP1A1 and GST (two enzymes regulated by AhR) mRNA and protein expression were increased at the higher doses of BPA and with E_2. Curiously, the 2 μg/kg dose of BPA did not increase AhR and related protein expression, although both lower (0.02 μg/kg d) and higher (200 and 20,000 μg/kg d) doses were effective.

In a separate paper with no comparison estrogen, BPA was generally found to increase RAR and RXR expression over the dose range (Nishizawa et al., 2005a). A third paper reported that BPA generally decreased RAR and RXR expression in brain and gonads at the 2 μg/kg d dose (Nishizawa et al., 2003). Using this same dose, nuclear hormone receptors in placentas were also examined at GD 18.5 (Imanishi et al., 2003). The hypothesis centered on possible changes in nuclear receptors using a microarray panel of 20 nuclear receptors and 7 non-nuclear receptors. A sex-dependent pattern of up and down regulation was seen for several receptors, including progesterone receptor, estrogen receptor beta, steroidogenic factor 1, and alpha-fetoprotein mRNA. This work demonstrates that important developmental pathways in the embryo and placenta are impacted by BPA after oral administration to the dam at doses less than 1 mg/kg d. The exact series of events involved and the consequences for embryonic development have not been explored.

Gene expression changes in an important patterning gene were also indicated in a study by Smith and Taylor (2007). BPA injected i.p. in mice during organogenesis led to higher expression of the Hoxa10 protein in the uterus of immature (2-week old) and mature (6-week old) female offspring. This effect persisted in a small group of the immature mice that were ovariectomized prior to examination of Hoxa10, indicating that the enhanced gene expression was not secondary to higher estrogen stimulation of the uterus. E_2, DES, MXC, and a number of other xenoestrogens have been shown to alter Hoxa10 regulation during development.

Three papers have looked at changes in embryo/fetal DNA methylation due to BPA exposure. Changes in methylation are another pathway by which gene expression can be altered in the developing embryo/fetus. Changes in DNA methylation of the Agouti gene during embryogenesis produce different coat colors in adults. Dolinoy et al. showed that BPA treatment (50 mg/kg diet) resulted in a larger percent of offspring with yellow

coat color (Dolinoy et al., 2007). In another study, mouse embryonic forebrain was examined for methylation after maternal BPA exposure (Yaoi et al., 2008). Age specific (GD 12.5, 14.5) changes in methylation status were identified. Follow-up cloning showed that DNA with altered methylation was located in CpG islands (CGI) in gene promoter regions, a common site for the alteration of gene expression by methylation. A change in gene expression was confirmed for two of these genes, Vps52 and LOC7235, two proteins involved in membrane protein transport. This same BPA treatment was shown to alter forebrain morphology in other papers from this group (Nakamura et al., 2006, 2007b).

Postnatal Endpoints

Postnatal growth as indexed by body weight. Low level (<4 mg/kg d) prenatal BPA exposures have been associated with greater postnatal weights in immature offspring while higher BPA exposures (>20 mg/kg d) can produce growth retardation (Table 5). Greater postnatal weight gain at low doses may be relevant to underlying metabolic disorders, although body weight is a relatively crude reflection of altered metabolism.

As mentioned previously, when blastocysts exposed in vitro to BPA were transferred to the uterus of pseudopregnant mice, pregnancy and birth parameters did not differ from controls, but offspring were heavier at weaning (Takai et al., 2001). The weight differential was 39% for 1 nM exposure and 34% for the 100 μM exposure. This finding can be taken to suggest that the effects of developmental BPA on postnatal weight are not necessarily mediated by BPA effects on the maternal system, since the blastocysts were exposed ex utero.

This finding of significantly greater weaning weights after BPA exposure of preimplantation embryos is similar to a finding by Howdeshall et al. after exposure of postimplantation embryos (GD 11–17) to low doses (2.4 μg/kg d) of BPA in utero. Previous research had shown that estrogen/testosterone levels in fetuses depend to an extent on whether they are positioned next to male or female fetuses in the uterus. In this experiment the weaning weight differential was about 10% but varied depending on proximity to a female fetus in utero (Howdeshell et al., 1999; Howdeshell and vom Saal, 2000).

Two other studies examined postnatal body weight after longer periods of developmental exposure to low dose BPA via drinking water (Table 5). Rubin et al. (2001) exposed rats from GD 8 to PND 16 and reported higher weights in the BPA-exposed group beginning at birth. The higher weights were more persistent in female when compared to male offspring extending through PND 110, and were similar or less in the high than in the low dose group. Another study (Miyawaki et al., 2007) exposed mice from GD 10 to PND 30 and found greater body weights in the BPA-treated juvenile mice compared to controls. This effect was sex-dependent in that females were more affected at lower doses. Body weights were positively correlated with adipose tissue weight in both sexes, suggesting that greater weights were secondary to greater fat depots. Adipose tissue weight was in turn correlated positively with serum leptin, serum lipids, and glucose in females but not males. Statistical conclusions of these studies (Rubin et al., 2001, Miyawaki et al., 2007) are limited by the lack of litter-based statistics, the small number of dams, and the large litter sizes. Use of the litter as the unit for statistical analysis is an important consideration in developmental toxicity studies where treatment is administered to the dam. However, the general pattern of results is consistent with the findings of Takai and Howdeshall using prenatal exposure and litter-based statistics. There is also a recent study with early neonatal BPA exposure (PND 0–3) in rats which found greater weights in treated than control males on PND 68 (Patisaul and Bateman, 2008). That study used a dose of 50 μg/kg d by s.c. injection.

Consideration should be given to possible contradictory findings in other studies with BPA exposure. Although a large number of studies have administered BPA during pregnancy to rats and mice, some used higher doses (>10 mg/kg d) and many others did not report on weight growth. Table 5 includes studies with doses >10 mg/kg d which reported lower postnatal body weight in BPA-exposed groups. Some studies used only prenatal exposure, while some used combined prenatal/postnatal exposures.

Immune system. Immunotoxicity is a recently established research area of BPA developmental toxicity. Two studies with prenatal exposure and two studies with combined prenatal and postnatal exposure are available for review (Table 6). All studies used mice and evaluated immune function after weaning or in adults. The studies used oral dosing and the lowest effective doses were in the <50 μg/kg d range.

Hypotheses concerning BPA developmental immunotoxicity were based on:

- The known immune-modulating effects of estrogen.
- The demonstration that BPA could influence immune response endpoints in adult rodents (Richter et al., 2007; Willhite et al., 2008).

Mice exposed to BPA either as adults or in utero demonstrate exaggerated immune response. Mice exposed during early embryogenesis to BPA in drinking water had an enhanced cytokine and inflammatory response to antigen after infection with the protozoan Leishmania major (Yan et al., 2008). The number of specialized T-cells that regulate immune responses ($CD4^+$ $CD25^+$ T-cells) was reduced, a finding that is potentially relevant to hypersensitivity disorders like allergy and asthma. A similar hypersensitivity after immunization with protein antigen has been shown after prenatal BPA exposure by another laboratory (Yoshino et al., 2004). This study also presented data indicating no effect of the BPA exposure on litter size, sex ratio, or adult body weight. A third study using extended developmental exposure (prenatal and postnatal) similarly found reduced numbers of the specialized regulatory T-cells and increased antibody production in mice challenged as adults (Ohshima et al., 2007). This study measured serum BPA in dams and offspring at the end of the exposure period and reported that they were approximately 10 times the concentrations seen in humans.

Linking these immune system findings to possible epigenetic mechanisms, proteomic evaluation of spleen and thymus from 3- and 7-week-old mice exposed to BPA during development showed significant dose-dependent up or down regulation of seven proteins,

Table 6
BPA Developmental Immunotoxicity Studies

Reference	Species Sex	Route	Exposure period	Dose LOAEL	Findings
Prenatal					
Yoshino et al. (2004) NTP-A	DBA mice Male and female	Oral, oral instillation	17 days, beginning 1 day before mating	0, 3, 30, 300, and 3000 μg/kg d LOAEL 30 μg/kg d	↑ antibody production; ↑ splenic cytokine response
Yan et al. (2008)	BALB/C, C57Bl/6J mice male	Oral, drinking water	3 weeks beginning 2 weeks premating	0, 1, 10, and 100 nM 0, 0.03, 0.3, and 31 mg/kg d LOAEL 0.3 μg/kg d	↑ foot pad swelling; ↑ splenic cytokine response; ↓ $CD4^+CD25^+$ splenic lymphocytes
Prenatal and postnatal					
Ohshima et al. (2007)	Mice, WT and Tg, BalbC background, male	Oral, diet	Gestation and lactation	0, 0.1, and 1.0 ppm 0, 10, and 100 μg/kg d[a] LOAEL 10 μg/kg d	↑ cell proliferation; ↑ antibody production; ↓ $CD4^+CD25^+$ splenic lymphocytes
Yang et al. (2009)	ICR mice	Oral, drinking water	GD 7–PND 21	0, 9, and 171 mg/kg d No statistics reported	↑ ApoA1, DPP111 and VAT1 protein; ↑ ApoA1 mRNA in liver, spleen and thymus

WT, wildtype; Tg, transgenic; BPA, Bisphenol A; GD, gesatation day; PND, postnatal day.
[a]Estimated by OEHHA.

one of which (apo-A1) also showed both increased protein and mRNA expression on follow-up (Yang et al., 2009). Apo-A1 is involved in tissue cholesterol efflux and may have a role in regulating cytokine production. However, the proteomics study was carried out in a higher dose range than the immune response studies.

Sex differentiation of genital morphology. BPA effects on maturation of the reproductive tract have been studied primarily using exposures between birth and puberty. Some of the studies of sex differentiation of genital morphology using exclusively prenatal exposure (Table 7) are reviewed here, focusing on anogenital distance (AGD). In rodents, AGD is a marker for other abnormalities of genital differentiation (Welsh et al., 2008). In humans, AGD (as measured by anterior anoscrotal distance) was shorter in boys with hypospadias or cryptorchidism as compared to boys with normal genitalia (Hsieh et al., 2008). Investigators have also demonstrated induction of hypospadia in mice exposed to the estrogenic chemical benzophenone in utero (Hsieh et al., 2007). Similarly, girls androgenized due to congenital adrenal hyperplasia have longer AGD than controls (Bongiovanni, 1962). This suggests that AGD can be an index of a more pervasive disruption of sexual differentiation in humans.

AGD has most often been studied as a marker of secondary sex differentiation after prenatal BPA exposure of laboratory rodents. AGD is defined as the distance between the anus and genital papilla (penis or clitoris). AGD is greater in males than females beginning in the fetal period due to the action of endogenous androgens, specifically dihydrotestosterone. There is some evidence for the effects of BPA at the androgen receptor (AR). BPA binds and inhibits AR activation with an IC_{50} in the micromolar range (Paris et al., 2002; Bonefeld-Jorgensen et al., 2007; Vinggaard et al., 2008). Exogenous estrogenic agents can also affect AGD. Estrogen (E_2, 2 mg/kg, s.c. injection) during organogenesis (GD 11–14) produced a dramatic decrease of AGD in male mouse fetuses at term (Gupta and Goldman, 1986). E_2 induces AR expression in the genital tubercle, which is typically higher in females than in males in this area (Agras et al., 2006).

AGD measurement can be operationalized somewhat differently depending on how the animal is restrained for the measurement, the measuring tool, etc. (Vandenbergh and Huggett, 1995). AGD can also be measured internally at necropsy.

AGD is highly correlated with body size within sexes. Thus, a corrected version of AGD is usually reported as a ratio (AGD/bw, AGD/$bw^{1/3}$), or body weight is used as a covariate in statistical analyses.

Four studies reported a *longer* AGD (masculinized) in offspring after prenatal BPA exposure (Gupta, 2000; Honma et al., 2002; Tyl et al., 2002b, 2008a) (Although the multigeneration studies included prenatal and postnatal exposure, changes in the AGD on PND 0 could only be attributed to prenatal exposure). One study reported *shorter* AGD in male rats after prenatal BPA (Talsness et al., 2000b), while a second study using similar doses and time of exposure, but with a smaller sample size, found no effect (Tinwell et al., 2002). The Talsness et al. study used a 50 mg/kg d dose, higher than those in the Gupta and Honma et al. studies. Further, the administration was by gavage, potentially producing a higher peak exposure than in the multigeneration studies. In the Gupta study, the comparison estrogen DES produced *longer* AGDs at lower doses and *shorter* AGDs at higher doses.

Two additional indices of morphological sex maturation, preputial separation and vaginal opening, were measured in some of the studies with exclusively prenatal exposure (Table 8). The Honma et al. and Tinwell et al. studies used litter-based statistics, but the Talsness et al. study was not obviously litter-based. Varying designs and doses preclude integration of these studies.

Table 7
Effects on AGD After In Utero Exposure to BPA

Reference	Species Time of exposure Route of exposure Doses group size	Age at assessment Measure Sexes studied Comparison estrogen	Findings
Rat studies			
Talsness et al. (2000b) NTP-A	SD rats GD 6–21 Oral, gavage 0, 0.1, and 50 mg/kg d $N = 18, 20$	PND 3, 15, 21 AGD, AGD/$BW^{1/3}$ Male and female EE	Shorter, males, 50 mg/kg d, PND 15, 21; adjusted and unadjusted 0.1 mg/kg d shorter PND 15, 21. unadjusted; EE longer PND 15, 21
Tinwell et al. (2002) NTP-A EU (2008)	SD, Wistar rats GD 6–21 oral, gavage 0, 0.02, 0.1, and 50 mg/kg $N = 7$	24 hr after birth AGD Male and female EE	No effect; not adjusted; no effect of EE
Tyl et al. (2002b) NTP-A EU (2008)	SD rats, three-generation Premating-birth oral, diet 0, 0.001, 0.02, 0.3,5, 50, and 500 mg/kg d 1, 20, and 300 μg/kg d 5, 50, and 500 /kg d $N = 26–30$	PND 0 AGD Male and female No comparison estrogen	Longer in F_2 females; 4 doses; Not adjusted No effect males
Mouse studies			
Gupta (2000) NTP-A NTP-B EU (2003)	CD-1 mice GD 16–18 "Fed," probably o.i. 0 and 50 μg/kg d $N = 15$/group	PND 3, 21 AGD/BW Male DES	Longer in males DES, longer at low dose, shorter at high dose
Honma et al. (2002) NTP-A EU (2008)	ICR mice GD 11–17 Injection, s.c. 0, 2, and 20 μg/kg d $N = 10$	PND 22, 60 AGD/BW: Male and female DES	Longer in females PND 22; longer in males PND 60; Similar trends DES
Tyl et al. (2008a) NTP-A EU 2008 (cited as Tyl et al., 2007)	CD-1 mice, 3-gen Premating-birth oral, diet 0, 0.003, 0.03, 3.5, 50, and 600 mg/kg d $N = 55$ control, 19–25 BPA	PND 0 AGD, AGD adjusted Male and female E_2	Longer F_2 females 0.003 mg/kg d dose, unadjusted and adjusted by covariance No E_2 effect

o.i., oral instillation, BPA, bisphenol A; AGD, anogenital distance; PND, postnatal day; GD, gestation day; SD, Sprague–Dawley; EE, ethinyl estradiol.

Table 8
Other Findings on Morphological Sex Maturation in Studies With Prenatal BPA[a]

Reference	Findings
Talsness et al. (2000b) NTP-A	AGD shorter PPS later 0.1 mg/kg d Testes descent later 0.1 mg/kg d
Honma et al. (2002) NTP-A EU (2008)	AGD longer VO earlier 20 mg/kg d
Tinwell et al. (2002) NTP-A EU (2008)	AGD no BPA effects VO Wistar rats later 50 mg/kg d); no effect in SD rats PPS no BPA effect; Testes descent not studied; VO earlier EE

[a]AGD, anogenital distance; VO, vaginal opening; PPS, preputial separation; EE, ethinyl estradiol; SD, Sprague–Dawley; BPA, bisphenol A.

Brain and behavior

Sex-differentiated brain and behavioral endpoints: Exposures during the late fetal and early postnatal period are known to be most relevant for disruption of sexual differentiation of the brain in rodents. Thus, few BPA animal studies in this area use exclusively prenatal exposures. Division of exposure into prenatal and postnatal periods is meaningful in terms of maternal

mediation of effects that might occur, e.g. by maternal physiology in the gestational period, and maternal behavior and nutritional support via lactation in the postnatal period. However, it is less relevant to brain development. Brain development that takes place prenatally in humans largely occurs postnatally in rats and mice. Sexual differentiation of the brain occurs primarily in the second/third trimester in humans and nonhuman primates, but primarily in the neonatal period in rats and mice. In this section, studies with prenatal exposure are integrated along with closely related studies with pre- and postnatal exposure since all such studies may be relevant to potential risks for adverse effects on brain development in humans from in utero exposure.

Editorial comment in the BPA literature has suggested that studies of sexual differentiation of the brain in rodents cannot be generalized to humans because this process is mediated by estrogen in rodents and by androgen in primates (Dixson, 1998; Li et al., 2008; Longnecker, 2009; Sharpe, 2010). However, reviews of sexual differentiation of the brain report on wide variations within and between mammalian species and a role for many steroid hormones in diverse aspects of the process of brain sexual differentiation (Simerly, 2002; Wilson and Davies, 2007).

Sex differentiation of rodent brain morphology focuses on specific structures. Some sex-differentiated structures of rodent brain studied in connection with BPA are:

- AVPV, anteroventral periventricular nucleus of the hypothalamus, regulates gonadotropin releasing hormone, which in turn controls gonadal function. Th (tyrosine hydroxylase) is a marker for dopaminergic neurons. Neurons expressing both Th and ERα are seen only before puberty and are thought to regulate gonadotropin releasing hormone at this time. Size: ♀ > ♂
- BST, bed nucleus of the stria terminalis, is implicated in gender identity in humans and in stress response in rodents, associated with neurons expressing corticotrophin releasing hormone. The stria terminalis connects the amygdala with hypothalamic and thalamic areas. Size: ♂ > ♀
- Locus coeruleus is a structure in the brainstem that is the origin of noradrenergic neurons of the brain and is a target of human pharmacotherapy agents such as modafanil and reboxitine for anxiety and panic disorders. Size: ♀ > ♂
- SDN-POA, sexually dimorphic nucleus of the preoptic area of the hypothalamus, regulates male reproductive behaviors. Calbindin influences apoptosis of cells in this area in males, which produces the sexual differentiation in size. Size: ♂ > ♀

Sex differentiation of rodent behavior includes nonreproductive behaviors as well as reproductive behaviors. In the most strictly defined framework, behavior is sex-differentiated if it is ''fundamentally and permanently'' different between sexes and is known to be determined developmentally by gonadal hormones (McCarthy and Konkle, 2005).

Some sex-differentiated behaviors in rodents are:

- Mating behavior.
- Parental behavior.
- Social interaction (including aggression and play).
- Affective behavior (anxiety).
- Spatial learning and memory.

There is a large and fascinating animal behavior literature concerning sex differences in behavior in various species. This literature is valuable in providing perspective and bridging the gap between rodent endocrine disruption studies and human public health issues. Although differences between rodents and primates are known (Li et al., 2008; Longnecker, 2009), there are also differences just as large within rodent and primate groups.

There are two basic approaches to identifying an effect on sex differentiation of brain or behavior:

- Sex differences occur in controls, but not in treated groups.
- Treatment effects occur in one sex but not the other.

In the latter approach, a sex-differentiated behavior is usually studied. Some studies reviewed below used the former approach (compare sexes within treatment groups; see later sections), and some used the latter approach (compare treatment groups within sexes; see later section), making findings difficult to compare on an agree/disagree basis when these different approaches are used.

In other scenarios, BPA effects on a behavioral measure may be seen in both sexes after prenatal exposure (later section). These scenarios are relevant to developmental neurobehavioral toxicity although they do not support hypotheses concerning BPA effects on sex differentiation of the brain and behavior.

Studies with 15–1500 μg/kg d BPA exposures in Wistar rats: BPA doses of 1.5 mg/kg d during prenatal/postnatal development did not affect sexual differentiation of Wistar rats as reflected in estrus cycles, sperm count, AGD, vaginal opening, serum hormones, the volume of the brain area associated with reproductive tract differentiation (SDN-POA), or mating behavior (Kubo et al., 2001, 2003) (Table 9). However, the volume of the locus coeruleus, another sex-differentiated brain area associated with sex differences in affective behavior (anxiety and depression), was larger in control females than males, but smaller in BPA-treated females than males. This finding was consistent with the absence of sex difference in the BPA-treated offspring for measures taken in a novel environment and in response to shock. Female rodents typically show less emotional response in these tests. Because sexual differentiation of the locus coeruleus, which occurs prenatally in the rat, appeared to be the endpoint most sensitive to BPA, a third study was undertaken looking specifically at prenatal exposure and at an expanded set of behavioral tests that depend on this brain area (Fujimoto et al., 2006).

The volume and neuron number of the locus coeruleus is sexually dimorphic in rodents, being larger in females than in males. This sexual dimorphism occurs under the influence of prenatal hormones, and AR is an important mediator (Guillamon et al., 1988; Garcia-Falgueras et al., 2005). In contrast, the sexual differentiation of the SDN-POA occurs later, on GD 18 through PND 5 (Rhees et al., 1990a,b). Behavioral assessments used in rats to assess locus coeruleus function include activity in a novel open field and avoidance of shock. Female rats typically show

Table 9
Studies of Sexual Differentiation of the Brain and Behavior in Wistar Rats Exposed Via Drinking Water to Low Doses of BPA ($\leq$1.5 mg/kg d)

Study	Exposure time/dose	Endpoint	BPA effect
Kubo et al. (2001)	GD 0–PND 21	Reproductive organ weights	No effect
NTP-A	0 and 1.5 mg/kg d	Hormones (LH, FSH, and E_2/T)	No effect
EU (2008)	Drinking water	Brain: SDN-POA, Volume	Cont: sex diff
			BPA: sex diff
		Brain: Locus Coeruleus	Cont: sex diff
		Volume, cell #	BPA: no sex diff
		Behavior: Exploratory	Cont: sex diff
			BPA: no sex diff
		Behavior: Passive avoidance	Cont: sex diff
			BPA: no sex diff
Kubo et al. (2003)	GD 0–PND 21	Reproductive organ weights	
NTP-A	0, 30, and 300 μg/kg d	Hormones (LH, FSH, E2/T)	No effect
EU (2008)	Drinking water	Sperm parameters	No effect
	Comparison estrogens:	Estrus cycles	No effect
	Resveratrol, DES	Anogenital distance	No effect
		Testes descent	No effect
		Vaginal opening	No effect
		Behavior: male mating	No effect
			No effect
		Brain: SDN-POA, volume	Cont: sex diff
			BPA: sex diff
		Brain: Locus Coeruleus,	Cont: sex diff
		volume, cell#	BPA: no sex diff
			30, 300 μg/kg d
		Behavior: Exploratory	Cont: sex diff
			BPA: no sex diff
			30, 300 μg/kg d
Fujimoto et al. (2006)	GD 13–PND 0	Behavior: Exploratory	Cont: sex diff
NTP-A	0 and 15 μg/kg d		BPA: no sex diff
EU (2008)	Drinking water	Behavior: Passive avoidance	Cont: sex diff
			BPA: sex diff
		Behavior: Forced Swim	Cont: sex diff
			BPA: no sex diff
		Behavior: Elevated Plus maze	Cont: no sex diff
			BPA: no sex diff

BPA, bisphenol A; PND, postnatal day; DES, diethylstilbestrol; GD, gestation day.

less anxiety and depression in these tests. At higher doses with prenatal and postnatal BPA exposure, exploratory behavior in an open field and passive avoidance of shock failed to show anticipated sex differentiation (Kubo et al., 2001, 2003). With the lower dose and exclusively prenatal BPA exposure, sex differentiation did not occur for rearing, one of the open field measures which was sex-differentiated in controls (Funabishi et al., 2006). However, passive avoidance behavior was sex differentiated after the low dose BPA treatment. Two additional tests in the low dose prenatal experiment were persistence of swimming in inescapable water, and time spent in open versus enclosed areas of an elevated plus maze. Significant sex differentiation of elevated plus maze behavior was not demonstrated in either control or BPA groups, while sex-differentiation of struggling behavior in the forced swim test seen in controls (more struggling in females) was eliminated in the BPA group. Immobility in the forced swim test was increased by BPA, and this effect was also significant in males only, although immobility was not sex-differentiated in controls.

A number of other studies have examined sexual differentiation of the brain using either postnatal or combined prenatal and postnatal BPA administration (Table 10). Some of these studies compared sexes within treatment groups and some compared treatment groups within sexes. They suggest that size of brain areas that are typically sex differentiated in rodents (SDN-POA and AVPV) is not influenced by BPA, but sex differentiation in terms of the number of phenotypically sex-differentiated neurons can be affected. An exception is the volume of the locus coeruleus, which has been shown to be affected by developmental BPA exposure as discussed above.

Studies with 40 μg/kg d BPA exposure in SD rats: This series of studies includes five papers using perinatal (pre and postnatal combined) exposures (Farabollini et al., 1999; Dessi-Fulgheri et al., 2002; Adriani et al., 2003; Della Seta et al., 2005; Porrini et al., 2005), two papers which used cross fostering to identify exclusively prenatal effects (Aloisi et al., 2002; Farabollini et al., 2002), and one paper that used only postnatal exposure (Della Seta et al., 2006) (Table 11). In this model, BPA was administered by micropipette into the mouth (oral instillation, o.i.) to Sprague–Dawley (SD) rats. The initial studies used a short-term higher dose, as well as a long-term lower dose of 40 μg/kg d. Some studies included a comparison estrogen (Della Seta et al., 2006). Two other papers were directed at

Table 10
BPA Effects on Sex Differentiation of the Rat Brain

Study Exposure period (dam/pup) Dose Comparison estrogen	Structures/measures age at assessment	Findings
Funabashi et al. (2004) Wistar rats Pregnancy/lactation (dam) 2.5 mg/kg d drinking water NTP-B EU (2008)	SDN-POA: CRH immunoreactive neurons BST: CRH immunoreactive neurons Assessed at 4–7 months of age	Control: sex diff BPA: sex diff Control: sex diff BPA: no sex diff
Kubo et al. (2003) Wistar rats GD 0–PND 21 (dam) 30 and 300 μg/kg d drinking water DES comparison NTP-A EU (2008)	SDN-POA, volume, cell ♯ Locus coeruleus, volume, cell ♯ Assessed at 14 weeks of age	Control: sex diff BPA: sex diff Control: sex diff BPA: no sex diff
Kwon et al. (2000) SD rats GD 11–PND 10 (dam) 320 /kg d Gavage DES comparison NTP-A EU (2008)	SDN-POA volume, Assessed at 10 days of age	Females: no BPA effect
Nagao et al. (1999) SD rats PND 1–5 (pup) 300 μmg/g s.c. = 3 mg/kg bw-d NTP-A	SDN-POA volume, Assessed at 14 weeks of age, males only	Males: no BPA effect
Takagi et al. (2004) SD rats GD 15–PND 10 (dam) 0, 60, 600, and 3000 diet 0, 6, 65, and 308 mg/kg d NTP-A EU (2008)	SDN–POA volume, adult males and females Assessed at 21 days of age and 11 weeks of age	Males: no BPA effect Females: no BPA effect

None of these studies used exclusively prenatal exposure.
PND, postnatal day; SD, Sprague–Dawley; DES, diethylstilbestrol; GD, gestation day; BPA, bisphenol A; CRH, corticotropin releasing hormone.

the examination of estrogen-related brain mechanisms (Facciolo et al., 2002, 2005).

The research focused on BPA effects on social and reproductive behaviors, using a variety of potentially sensitive assays at the same dose level. Sex-differentiated nonreproductive behaviors were also studied. In these experiments, sex differentiation was assessed by examining treatment effects within sexes. Behavioral evaluations occurred at different times in the life cycle including:

- PND 0–21 (preweaning) infancy;
- PND 21–28 juvenile;
- PND 29–60 pubertal/adolescent;
- PND 60+ adult.

The investigators used a dose level thought to be relevant to human exposures, below the dose of 50 μg/kg d considered to be without effect in several human risk assessments. They did not provide detailed information on pregnancy outcome, or examine reproductive tract morphology as the doses were below the expected threshold for these effects. Two studies from the series (Adriani et al., 2003; Della Seta et al., 2005) provided information on body weight. Using the mating-through-weaning dosing period, no effects were found on pup body weight on PND 2, 7, or 21 or on adult offspring weight (>70 days of age).

In the first paper in the series, social interaction was studied in juvenile/adolescent offspring (Dessi-Fulgheri et al., 2002). The BPA exposure was to the dam during pregnancy and lactation and interactions between treatment and offspring sex were considered. Juvenile social interaction endpoints sensitive to the BPA exposure included play directed to females, immature reproductive behavior, socio-sexual exploration, and social interest. As regards sex-differentiation of the treatment effect, immature reproductive behavior and social interest were not strongly sex differentiated in controls, and BPA had similar effects in both sexes, namely a decrease in the frequency of these behaviors. For socio-sexual exploration, the behaviors were more highly sex-differentiated and BPA affected males and females differently, increasing the frequency of behaviors at the low dose in females and decreasing the frequency of behaviors at the high dose in males. Play directed toward females was also highly sex-differentiated. BPA increased these behaviors at the low dose in females and at the high dose in males.

Further information on socio-sexual behavior (aggression and mating) in adult offspring was provided in a second paper (Farabollini et al., 2002) which used fostering to identify exclusively prenatal effects. This study used 72 offspring from 20 pregnancies, suggesting 3–4 rats per litter. Data were analyzed within sex.

Table 11
Studies of BPA Effects on Sex-Differentiation and BPA Sex-Specific Effects on Behavior Using 40 μg/kg d Dose by o.i. in SD Rats

Study	Exposure period dose	Endpoint age at assessment	Findings
Prenatal or postnatal exposure			
Farabollini et al. (2002)	Pregnancy or lactation	Socio sexual behavior, PND 100	↑ latency to intromission (pre)
NTP-A	(cross fostered)		↑ number of intromissions (post)
EU (2008)	40 μg/kg d o.i.		↑ genital sniffing (pre)
Aloisi et al. (2002)	Pregnancy or lactation	Pain response. PNW 22	↑ limb flexion (pre)
NTP-A	(cross fostered)		↓ paw jerk (post)
EU (2008)	40 μg/kg d o.i.		
Prenatal and postnatal exposure			
Farabollini et al. (1999)	Pregnancy and lactation	Exploration, (holeboard)	↓ head dipping
NTP-A	40 μg/kg d; or	anxiety (elevated plus maze)	↓ activity
EU (2008)	GD 14–PND 6	PND 85	↓ anxiety
	400 μg/kg d o.i.		
Dessi-Fulgheri et al. (2002)	Pregnancy and lactation	Juvenile social interaction,	↑ play directed to females
NTP-A	40 μg/kg d; or	8 factors derived from PCA of	↓ low intensity mating behavior
EU (2008)	GD 14–PND 6	behavior scoring	↓ sociosexual exploration males
	400 μg/kg d o.i.	PND 25, 35, 45	↑ sociosexual exploration females
			↓ social interest
Della Seta et al. (2005)	Pregnancy and lactation;	Maternal behavior	↓ duration licking grooming pups
NTP-A	40 μg/kg d o.i.	PND 3, 4; PND 8, 9	
Porrini et al.(2005)	Pregnancy and lactation;	Social behavior of female	↑ social/nonsoc explore
EU (2008)	40 μg/kg d	offspring	↓ play with males
NTP-A	o.i.	PND 35,45, 55	↓ social grooming
Adriani et al. (2003)	Pregnancy and lactation	Novelty preference PND 30–45	↓ females
NTP-A	40 μg/kg d o.i.	Activity PND 35–45	↑ males and females
EU (2008)		Impulsive behavior "adult"	↓ males and females
		Amphetamine response "adult"	↓ males
Della Seta et al. (2006)	PND 23–30	Male social and sexual behavior	↓ latency to intromission
NTP-A	40 μg/kg d o.i.	PND 45, 90	
EU (2008)			

PCA, principal components analysis; o.i., oral instillation; PND, postnatal day; GD, gestation day; BPA, bisphenol A.

In males, the aggression test demonstrated significantly more defensive and less aggressive behavior during agonistic encounters in the prenatally treated BPA group. The same pattern was seen for the aggression test in females, but comparisons were not statistically significant. No BPA effects were seen in the sexual orientation test (time spent near rat of the opposite or same sex). The latency to intromission (insertion of penis into the vagina during mating) was increased in prenatally exposed males, while the number of intromissions was increased in postnatally exposed males. Additionally, the duration of genital sniffing was increased in prenatally BPA-treated males compared to controls. In females, the latency to approach males and the frequency of lordosis were greater in BPA-treated females tested during the proestrous/estrous stage of the estrous cycle (when mating behaviors usually occur). Pre- and postnatal BPA groups were combined for the analysis since they did not differ statistically; thus, time of exposure was not a factor in the analysis and exclusively prenatal effects could not be identified for the estrous cycle endpoint.

Although not part of this series of studies, a recent investigation of aggression in male Long–Evans rats exposed developmentally to BPA has been conducted (Patisaul and Bateman, 2008). This study used a BPA dose of 50 μg/kg d similar to the 40 μg/kg d dose of the Farabollini study. However, a brief postnatal exposure by injection to the pups was used (PND 0–3) and no effect on aggression was found when the males were adults.

Further studies of sexual behavior were also conducted with exposures in male juveniles prior to puberty (PND 23–30) (Della Seta et al., 2006). In this study, EE (0.4 μg/kg d) was used as a positive control. When tested as adults (>90 days of age) the direction of mean differences from control was similar for the BPA and EE-treated groups, although statistical significance in post hoc tests was usually reached only in the EE group. Latency to intromission was the only endpoint significant for the BPA group in post hoc comparisons. EE and BPA groups had a shorter latency to mount and to intromission (not all rats ejaculated), and a higher frequency of intromissions and intromissions per mount. Duration of genital sniffing, however, was lower in the EE and BPA groups. In separate groups, plasma testosterone (T) and estradiol were measured both during the pubertal treatment (PND 37) and in adults (PND 105) (groups of 5–8). E_2 was not influenced by treatment but T was lower than control in both the EE and BPA groups at both ages, with the BPA group having the lowest mean, a major finding of the study.

A final reproductive behavior study in this model was maternal behavior. Using the exposure period beginning premating through lactation, maternal behavior was evaluated at PND 3, 4 and PND 8, 9 (Della Seta et al., 2005). The analysis reported generally reduced behavior in terms of duration and frequency of licking–grooming,

anogenital licking, and arched-back posture; the change in the duration of licking–grooming was significant by ANOVA at $p<0.05$. Nest building was not affected. This study is not relevant to developmental toxicity but adds information on BPA effects on sex-differentiated behaviors.

Several papers in this series looked at sex-differentiated nonreproductive behavior. A sex-differentiated behavior, response to pain, was assessed in BPA-treated adult offspring (Aloisi et al., 2002). Prenatal/postnatal dosing and cross fostering were used as in Farabollini et al. (2002). In the test, formalin was injected into the paw resulting in three responses known to be sex-differentiated and influenced by exogenous estrogen (Aloisi et al., 1994, 1997; Aloisi and Ceccarelli, 2000; Aloisi, 2003).

- Withdrawal of the paw (limb flexion); increased by estrogen.
- Licking of the paw; increased by estrogen.
- Shaking the paw (paw jerk); decreased by estrogen.

The prenatal BPA-treated groups showed greater pain response on variables that increase in response to exogenous estrogen (licking and flexing of the affected limb). Additionally, postnatal BPA decreased paw jerk response, which is also consistent with an estrogenic effect. Measures of general exploratory behavior were also obtained in this study, most of which were sex-differentiated. The data generally indicated reduced exploration in the prenatally exposed groups, consistent with the earlier study (Farabollini et al., 1999), but the BPA effect was not statistically significant.

In other studies, behaviors reflecting emotionality were considered. Adult offspring exploratory endpoints altered by developmental BPA in the first study (Farabollini et al., 1999) included head dipping in the hole board test (↓), and square crossing in the hole board exploration test (↓) as well as in the plus maze test (↓). There were some sex-specific effects. BPA-exposed males (but not females) had less indication of anxiety (greater percent entries into the open arm of the maze) compared to controls. In the second study (Adriani et al., 2003), behaviors reflecting serotonergic and dopaminergic systems were targeted: novelty preference (dopamine), impulsivity/reward discounting (serotonin), and response to amphetamine (dopamine). Most of the measures taken during the test were sex-differentiated, and showed sex by treatment interactions. Novelty preference was reduced in BPA-treated females only, and response to amphetamine was reduced in males only. The impulsivity measure was not sex-differentiated and showed an effect of BPA with the sexes combined (less impulsivity in the BPA group). Responding during the intertrial interval of the impulsivity task was sex-differentiated and showed a BPA effect (increased responding) in males only.

Although not part of this series of studies, a recent investigation in male Long Evans rats exposed developmentally to BPA also found reduced anxiety in the elevated plus maze (Patisaul and Bateman, 2008). This study used a BPA dose of 50 μg/kg d, similar to the 40 μg/kg d dose of the Farabollini study. Here a brief postnatal exposure by injection to the pups was used (PND 0–3).

Changes in brain have also been studied in this model. These studies focused on somatostatin receptors, neuropeptide receptors that develop a sex-differentiated pattern of expression in the brain under the influence of estrogen (Table 11). Somatostatin inhibits the secretion of various hormones that promote growth such as growth hormone, glucagon, insulin, thyrotropin, and gastrin. Somatostatin receptors colocalize on the cell membrane with the membrane estrogen receptor (GP 30), as well with a receptor for the inhibitory neurotransmitter GABA (GABAα receptor). Somatostatin receptor genes have an estrogen response element and BPA was shown to induce somatostatin receptor subtype 3 (sst3) expression in hypothalamic areas in a manner that interacted with the effect of specific GABA agonists (Facciolo et al., 2005). In other studies, BPA was found to bind to sst2 in limbic areas of the brain of PND 10 and PND 23 rats (Facciolo et al., 2002). In both studies, dams were given the BPA treatments (40 or 400 μg/k/d) beginning before mating and continuing through lactation. Brain somatostatin receptors have also been shown to be influenced by treatment with atrazine, an estrogenic pesticide (Giusi et al., 2006).

Thus, in this group of studies, prenatal BPA exposure at 40 μg/kg dose in SD rats was reported to affect mating and aggressive behavior in male rat offspring (both decreased relative to controls) and alter pain response in both male and female offspring (increased relative to controls). These effects resulting from prenatal exposure occur in the context of more extensive changes in exploratory, social, and reproductive behavior and changes in brain after prenatal/postnatal exposure at the same dose.

Studies with F344 rats: There are three studies of developmental BPA effects in F344 rat models, none of which used exclusively prenatal exposure (Table 12). These studies are valuable in investigating effects of developmental BPA exposure on behavior in a rat strain other than SD. Research has shown that inbred mouse and rat strains differ in their response to exogenous estrogen and estrogenic agents (Spearow et al., 1999, 2001; Spearow and Barkley, 2001) and that the F344 strain is more sensitive to estrogen than the SD strain in some assays.

One of the studies used the approach of assessing treatment effects within sexes (Negishi et al., 2003). A follow-up study at a lower dose tested only males (Negishi et al., 2004a). One study (Carr et al., 2003) examined a well-known sex-differentiated behavior, spatial learning, and memory, and assessed sex differences within treatment groups.

In the first study (Negishi et al., 2003), both juvenile (PND 28–34) and adult (PND 56–62) behavior were studied. BPA was reported to affect the behavior of males (but not females) on several endpoints: shock avoidance performance at both ages, grooming behavior in the adults, and time spent immobile during the dark phase of the daily diurnal cycle in juveniles. None of the behaviors affected by BPA were sex-differentiated behaviors in this study (no effect of sex in the ANOVA). In addition to different effects on males and females, low (4 mg/kg d), intermediate (40 mg/kg d), and higher (400 mg/kg d) doses of BPA had some qualitatively different effects. The low dose was most effective in inducing higher duration of grooming in adult males and

Table 12
Studies in Fischer 344 Rats With Prenatal/Postnatal Exposure

Study	Exposure	Endpoint	Effects
Negishi et al. (2003) NTP-A EU (2003)	0, 4, 40, and 400 mg/kg d GD 10–PND 20 Gavage $N = 8$–18/group	Spontaneous motor activity Exploratory activity Active avoidance learning	↑ % time immobile in dark phase, ♀ 40 mg/kg d, test before puberty ↓ % grooming, ♂, 4 mg/kg d, No sex effect ↓ avoidance, 4 mg/kg d, 8 weeks old, no sex effect
Negishi et al. (2004a) NTP-A EU (2003)	100 μg/kg d; GD 3–PND 20 Gavage $N = 9$, 10/group	Males only tested Spontaneous motor activity Exploratory activity Elevated plus maze Passive avoidance learning Active avoidance learning	No effect No effect No effect No effect ↓ avoidance, 15 weeks of age
Carr et al. (2003) NTP-A EU (2008)	100 and 250 μg/kg d PND 1–14 Gavage $N = 10$/group	Spatial learning	Cont: sex diff BPA: 100 μg/kg/d: no sex diff E_2: 72 μg/kg/d: no sex diff

PND, postnatal day; GD, gestation day; BPA, bisphenol A.

lower avoidance response of adult males. The intermediate and high doses affected avoidance response of juvenile males and the intermediate dose affected immobility in females.

Data on general toxicity were provided in this study. BPA influenced maternal body weight during pregnancy. Maternal organ weights at weaning were not affected with the exception of the thymus, which was lighter at the highest dose. Litter size was not affected but postnatal offspring weights were lower in some BPA-treated groups (see discussion under body weight above). The group size was $N = 8$–9 dams with litters culled to 8 at birth and $N = 8$–27 per group for offspring behavior tests. The figure captions suggest that all offspring were used for the juvenile activity test, and then some were selected for the avoidance test and others for the open field test. It was not clear whether litter-based statistics were used.

A follow-up study by this group (Negishi et al., 2004a) with a lower dose exposure (100 μg/kg d, GD 3–PND 20) used avoidance tasks shown to be sensitive at higher doses and a comparison estrogenic agent (nonylphenol). These tests were conducted in adult male offspring. The extended test battery included the elevated plus maze, an anxiety test. This test battery, as well as tests of exploratory behavior and spontaneous motor activity, were not found to be affected by the low dose BPA. Similarly, passive avoidance was not affected, but the BPA exposure led to poorer performance of the active avoidance task. This was similar to the result at the lowest previously tested dose (4 mg/kg d) but dissimilar to higher doses (40, 400 mg/kg d) which increased avoidance performance. Also, BPA-treated males failed to show hyperactivity in response to monoamine oxidase inhibitor injection as was shown by controls. Similar results were reported for the comparison estrogen, nonylphenol. Notably, BPA exposures were not limited to the prenatal period. This study demonstrates effects of BPA in a third rat strain (in addition to Wistar and SD, Tables 9 and 11), different effects of high and low doses, connection to monoamine systems in brain, and effects at doses less than 1 mg/kg d by the oral route.

The Carr et al. study in F344 rats focused specifically on spatial learning and memory, a cognitive behavior known to be sex-differentiated and influenced by developmental hormone exposures in both rodents and humans. Offspring were tested as adolescents (PND 34–38). Learning of this test was found to be sex-differentiated, with male controls performing significantly better than females on the last day of training. However, this sex difference in performance did not appear in the low dose BPA-treated offspring. At the higher dose (250 μg/kg) the sex difference was clearly seen. This study reported that BPA affects sex-differentiated behavior, and that effects differ depending on dose. E_2, the comparison estrogen, also led to an absence of sex difference in acquisition of the spatial task.

Studies with 10 μg/kg d BPA in CD-1 mice: Investigators who had previously studied the effects of other estrogenic agents (DES, MXC, and o,p'-DDT) on sexual differentiation of brain and behavior in mice (Palanza et al., 1999; Panzica et al., 2007) undertook similar studies with BPA. They used the oral instillation technique of administration and corn oil as a vehicle (Palanza et al., 2008). The 10 μg/kg dose was selected for use because it is the tolerable daily intake established for humans by the EU (European Commission Scientific Committee on Food, 2002). The focus of the studies was reproductive behavior (maternal behavior) as well as sex-differentiated exploratory and affective behaviors. The studies are outlined in Table 13.

Two studies used prenatal exposure (GD 11–18, GD 14–18) (Palanza et al., 2002b; Laviola et al., 2005) and one continued the exposure to the early postnatal period (GD 11–PND 8) (Gioiosa et al., 2007). General toxicity data were limited but no effects on dams' gestational weight, litter size, offspring weights at PND 3–15, or pup motor development (righting and cliff aversion) were seen in the first study (Palanza et al., 2002b).

The first study examined maternal behavior (Palanza et al., 2002b). There was no positive control (estrogenic agent) in this study, but a previous study by this group found a similar effect with MXC-treated females (Palanza et al., 2002a). BPA was found to decrease nursing

Table 13
Studies in CD-1 Mice With 10 μg/kg BPA Administered by Oral Instillation

Study	Exposure	Endpoint	Findings
Palanza et al. (2002a,b) NTP-A EU (2008)	GD 14–18; treatment in utero and/or when pregnant 10 μg/kg d o.i.	Maternal and nonmaternal behavior PND 2–15	↓ nursing behavior ↑ behavior away from nest In utero or when pregnant; in utero and pregnant not affected
Laviola et al. (2005) NTP-A EU (2008)	GD 11–18; $n = 10$–12 dams/group; MXC comparison (20 μg/kg d) 10 μg/kg d o.i.	Amphetamine conditioned place preference, PND 60	↓ place preference BPA and MXC females (not males)
Gioiosa et al. (2007) NTP-B	GD 11–PND 8 10 μg/kg d o.i.	Exploratory behavior: Time in open field Time in light area Time in center Time near home cage Elevated plus maze: Time in closed arms, entries into open arms Novelty preference test: Time in novel area Activity in novel area Risk assessment	Control: sex diff BPA: no sex diff Control: sex diff BPA: no sex diff Control: sex diff BPA: no sex diff

MXC, methoxychlor; BPA, bisphenol A; o.i., oral instillation; PND, postnatal day; GD, gestation day.

behavior in mice that were treated with BPA either in utero or during pregnancy as adults. This is in general agreement with a study of BPA effects on maternal behavior in rats (Della Seta et al., 2005). This study used 2–3 pups per litter per group in a split litter design with a total of 51 control F_1 females and 31 BPA-treated F_1 females. Each treatment group was further subdivided into two for the adult pregnancy treatment; exact group sizes for the four resulting groups were not given.

The second study looked at amphetamine-conditioned place preference in offspring of mice treated during gestation (Laviola et al., 2005). In this paradigm, an amphetamine injection at one of the three doses was administered to adult mice in one of the two distinct locations. Research has shown that mice will prefer the location where they experience the consequences of the amphetamine injection. Conditioned place preference is a sex-differentiated behavior, with males having a somewhat higher preference for the drug-related location than females. Female offspring of BPA-treated dams did not show conditioned place preference, indicating that the rewarding effects of amphetamine were not experienced. A similar lack of preference was seen in the MXC-treated females included as a positive control. BPA did not affect the place preference of males. This study used one male and one female pup per litter per dose, thus providing a litter-based statistical analysis.

The third study looked at sex-differentiated exploratory and affective behavior (Gioiosa et al., 2007). This study extended the exposure period into the first postnatal week and tested the offspring as juveniles and adults. Tests were selected that commonly show sex-differentiation. The majority of the endpoints measured in the three tests (open field, elevated plus maze, and novelty preference) demonstrated sex by treatment interactions, with comparisons in most cases supporting sex difference in the control but not the BPA group. An MXC-treated group showed a similar pattern of effects.

The results of this most recent study (Gioiosa et al., 2007) are consistent with previously reviewed studies in which developmental BPA at various doses was found to eliminate sex differences in open field exploratory behavior:

1500 μg/kg d (Kubo et al., 2001)
300 μg/kg d (Kubo et al., 2003)
30 μg/kg d (Kubo et al., 2003)
15 μg/kg d (Fujimoto et al., 2006)
10 μg/kg d (Gioiosa et al., 2007)

On the other hand, findings of Gioiosa et al. from the elevated plus maze test are not readily comparable to previously reviewed studies with the elevated plus maze because the previous studies did not evaluate sex differences:

- Only one sex was tested (Negishi et al., 2004a; Ryan and Vandenbergh, 2006; Patisaul and Bateman, 2008).
- No sex differences were detected in controls (Negishi et al., 2004a).
- Sex differences were not evaluated within groups (Farabollini et al., 1999).

However, the study by Farabollini et al. (1999) using the elevated plus maze did find an overall sex difference along with a significant interaction between treatment and sex. This analysis generally supports the findings of Gioiosa et al. with the elevated plus maze. There were no other studies using the novelty preference test.

The altered response to amphetamine in the Laviola et al. study is also consistent with studies in other models:

- Lack of hyperactivity response to monoamine oxidase inhibitor in male F344 rats (Negishi et al., 2004a).
- Reduced hyperactivity response to amphetamine in male SD rats (Adriani et al., 2003).

Two studies using only female rodents: Several studies of reproductive behavior (mating, aggression, and maternal care) reviewed above used only one sex. However, two studies looked more broadly at nonreproductive sexually differentiated behavior in females only.

A behavioral study used only female C57Bl/6 mice (Ryan and Vandenbergh, 2006). Developmental BPA (2 or 200 μg/kg d) and EE (5 μg/kg d) as a positive control were administered by oral instillation on GD 3–PND 21. The offspring ($n = 14/16$ litters per group, 1 female per litter) were ovariectomized prior to puberty. This would ensure that behavioral effects were mediated by BPA actions on brain, rather than BPA actions on ovary that indirectly affected behavior by changing hormone production. EE was effective in accelerating puberty, increasing anxiety (elevated plus maze and light dark box), and improving spatial memory (radial arm maze and Barnes maze). The high BPA dose had a similar effect on puberty and anxiety as did EE. While both EE and high dose BPA had some effects on spatial learning and memory, they were not clearly parallel.

Ryan et al. (2010) conducted a study in Long Evans rats that also used ovariectomy to remove the influence of ovarian hormone production on adult behavior after developmental BPA treatment (GD 7 to PND 18 by gavage). Additionally, EE injections were used to induce behavior prior to testing of female offspring as adults.

Three sex-differentiated behaviors were selected for study. The BPA doses were 2, 20, and 200 μg/kg d, while seven doses of EE (between 0.05 and 50 μg/kg d) were used. However, dose groups tested behaviorally and group sizes (based on litters) varied:

- Lordosis quotient: BPA 20, 200; EE 0.05, 0.15, 0.5, 1.5, 5, 15, 50 μg/kg d; $n = 1–7$/group.
- Activity: BPA 2, 20, 200; EE 0.5 5, 50 μg/kg d: $N = 5–13$/group.
- Saccharin preference: BPA 2, 20, 200; EE 0.5, 5, 50 μg/kg d: $N = 2–7$/group.

No BPA effects were found. EE did not influence the activity testing. EE effects on saccharin preference were limited to the highest dose, 50 μg/kg d, while both 15 and 50 μg/kg d affected the lordosis quotient. Notably, the 50 μg/kg d dose resulted in marked general toxicity in the experiment, resulting in 50% reduction in maternal weight gain, 18% reduction in implantation, 50% reduction in live pups per litter, >40% fetal/neonatal mortality, and reduction of live pups and female body weights at weaning (Howdeshell et al., 2008; Ryan et al., 2010). This general toxicity makes interpretation of behavioral effects difficult. The 15 μg/kg d also significantly affected maternal body weight gain and live pups per litter. BPA had no effects on general toxicity and reproductive outcome measures.

Although these studies tested hypotheses about sex-differentiated behavior, the evaluation of only one sex prevents the conclusion that sex-differences in behavior were/were not affected by BPA. Additionally, the small and varied group sizes and general toxicity in the Ryan et al. (2010) study limit conclusions related to this hypothesis.

Studies unrelated to sexual differentiation: Because of the known estrogenic action of BPA, most brain/behavior studies have focused on sexual differentiation, but some look more generally at neurobehavioral toxicity. Many of these studies were based on known biological actions of BPA other than its estrogenic action.

Thyroid-related effects: A series of studies from a laboratory in Japan (Table 14) were based on the observation that BPA is a thyroid hormone (TH) receptor antagonist (Zoeller et al., 2005; Zoeller, 2007) and can counteract the effects of TH on gene expression (Moriyama et al., 2002).

Bromodeoxyuridine can be used to label neural progenitor cells early in development and to track their migration and differentiation in the brain. Two studies used this technique to study prenatal BPA effects on cortical development (Nakamura et al., 2006, 2007a) (Table 14). An altered pattern of distribution of the labeled neurons in both the fetus and in weanling offspring was seen if pregnant dams were injected with 20 μg/kg BPA. The hypothesis was that BPA might bind protein disulfide isomerase, which acts as a storage protein for TH, increasing free TH. Gene expression of

Table 14
Studies of BPA Effects on Prenatal Mouse Brain Development

Study	Species	Dose route time	Assays	Findings
Nakamura et al. (2006) NTP-A EU (2008)	ICR/J mouse	20 μg/kg s.c. GD 0–14.5 or 16.5	Fetal neocortex GD 14.5, 16.5: Neurogenesis and migration; TH binding protein in neurons; genes regulated by TH and involved in neurogenesis	Accelerated neural differentiation and migration; upregulation of relevant TH regulated genes GD 14.5 including TH-binding protein disulfide isomerase
Nakamura et al. (2007a,b) NTP-B	ICR/J mouse	20 μg/kg s.c. BrdU injection on GD 12.5, 14.5, or 16.5	BrdU labeled cells in offspring cortex 3, 4, 5, 7, 8, and 12 weeks of age	Abnormal cortical location of GD 14.5 labeled neurons at 3 weeks of age; abnormal thalamocortical connections at 3 and 12 weeks of age
Yaoi et al. (2008)	ICR/J mouse	20 μg/kg s.c. GD–12.5 or 14.5	DNA methylation in offspring cortex GD 12.5, 14.5	Changes in methylation status and mRNA expression

TH regulated genes, including protein disulfide isomerase, was upregulated. Visualization of methylation of DNA fragments by the restriction landmark genomic scanning method showed that methylation status of a small specific set of sites was influenced by BPA treatment in a gestational age-dependent (GD 12.5, 14.5) manner. Identification of the location of the affected sites showed they were CGI in gene promoter regions. mRNA expression was examined at two of these sites and found to be altered in the GD 12.5 brains. These changes occurred in the absence of gross morphological or cytoarchitectural abnormalities. The dose for all studies was 20 μg/kg by s.c. injection.

The most recent advance using this model (20 μg/kg by s.c. injection during brain development) extended the treatment from GD 8 to PND 21 in rats and looked at functional changes in brain by examining electrophysiology in brain slices of cortex and striatum (Zhou et al., 2009). The authors reported failure of the normal progression of response to high frequency stimulation in this pathway, which converts from long-term potentiation to long-term depression during the third week of postnatal life. This finding was linked to dopaminergic systems and the work of other investigators on these systems. An action on dopamine systems through membrane estrogen receptor was discussed.

Effects on dopamine systems and interaction with drugs of abuse: Several hypothesis-testing studies (Table 15) were based on known influences of estrogen on dopamine systems in brain, which underlie reward and addiction processes, and the possibility that BPA interaction with estrogen receptors would secondarily modify dopamine systems.

Developmental BPA effects in response to drugs of abuse (morphine and methamphetamine) were studied in connection with BPA administered in diet to mice. Most of the studies used exposure from mating through gestation and lactation to weaning, but the most recent study (Narita et al., 2007) identified the prenatal period as the sensitive period for inducing this effect.

These studies found an enhancement of the effects of morphine and methamphetamine in male offspring of mice that had been treated with BPA during pregnancy and lactation.

Activity and conditioned place preference measures were used to assess enhanced morphine and methamphetamine effect. The effects were demonstrated at a dose of

Table 15
BPA Studies Related to Dopamine and Drugs of Abuse

Study	Species	Exposure	Endpoint	Findings
Suzuki et al. (2003) NTP-A EU (2008)	ddY mice, male	0, 2, 500, and 2,000 mg BPA/g diet 0, 0.2, 50, and 200 mg/kg bodyweight-d[a] GD 0–PND21	Methamphetamine conditioned place preference, methamphetamine stimulated activity Dopamine stimulated ^{35}SGTPγS; Dopamine membrane and vesicle transporter proteins, dopamine receptor D1	↑ preference ↑ activity ↑ activation No effect on DAT ↑ DRD1 mRNA
Mizuo et al. (2004a) NTP-A EU (2008)	ddY mice, male	0, 2, 500, and 2,000 μg BPA/g diet 0, 0.2, 50, and 200 μg/kg bodyweight-d GD 0–PND 21	Morphine conditioned place preference, Morphine stimulated activity Dopamine stimulated ^{35}SGTPγS	↑ preference ↑ activity No effects
Narita et al. (2006) NTP-A EU (2008)	ddY mice male, 7 weeks old	0, 0.03, 0.3, 3, 500, and 2,000 μg BPA/g diet 0, 3, and 30 μg/kg d 3, 50, and 200 mg/kg d GD 0–PND 21	Morphine conditioned place preference, Morphine stimulated activity Dopamine stimulated ^{35}SGTPγS	↑ preference ↑ activity ↑ activation Effects at 0.03 and 2000 μg BPA/g diet
Narita et al. (2007) EU (2008)	ddY mice, male	0 and 2,000 μg BPA/g diet GD 0–7 or GD 7–14 or GD 14–21 or PND 0–21	Morphine conditioned place preference, Morphine stimulated activity Dopamine stimulated ^{35}SGTPγS	Effects with dosing at GD 0–7 or PND 0–21
Tando et al. (2007) NTP-A EU (2008)	ddY mouse, male and female	3 μg/g, 8 μg BPA/g diet GD 0–PND 1	Dopamine neurons of substantia nigra and cortical neurons expressing calcium binding proteins 8–11 weeks of age	No effect on distribution of calcium binding proteins in cortical layers; Females: fewer Th (tyrosine hydroxylase) immunoreactive (ir) neurons in substantia nigra, low dose. Males: no effect on Th-ir neurons in SN

BPA, bisphenol A; PND, postnatal day; GD, gestation day.
[a]Doses in mg/kg body weight were calculated by the authors based on the assumption of that mice consume 10% of their body weight in food daily.

200 mg BPA/kg d for methamphetamine and 50 and 200 mg BPA/kg d for morphine (Suzuki et al., 2003; Mizuo et al., 2004a). Additionally, lower doses of BPA (0.003, 0.03, 0.3, 50, and 200 mg BPA/kg d) were investigated in connection with the morphine effect (Narita et al., 2006). The 200 mg/kg d dose was effective as was the lowest dose (3 μg/kg d), but intermediate doses had weaker or no effect, thus indicating a "U-shape" dose–response curve. A final study looked for sensitive periods for the effect using morphine (Narita et al., 2007). Four periods were considered: preimplantation GD 0–7, organogenesis GD 7–14, "parturition" GD 14–20, and lactation PND 0–20. The two periods when exposures were effective (using hyperactivity and conditioned place preference as the endpoints) were organogenesis and lactation. This study used the 200 mg/kg d dose, a higher dose than used in the studies of sex differentiation described above (Narita et al., 2007).

General toxicity data were not shown, but it was stated that there were no effects on weight or maternal behavior. One paper also reported the absence of effects on pup growth and birth rate (Narita et al., 2006). The number of dams treated and the number of male pups per litter used in each test were not stated. The group sizes ranged from 6 to 16. Age at testing was 7 weeks. Consistency in results across studies increases confidence in the results, although it is not clear that litter-based statistics were used.

The studies also directly assessed the dopamine systems in brain. The first study looked at dopamine receptors, dopamine transporters, and dopamine stimulated activation of G-protein signaling (Suzuki et al., 2003). The authors hypothesize that BPA does not act through classic estrogen receptors to produce low dose effect, but rather through the G-protein coupled membrane receptor, which can modify the actions of neurotransmitter receptors in the cell membrane. Effects of BPA on dopamine-mediated G-protein activation were confirmed in later studies (Mizuo et al., 2004a; Narita et al., 2006, 2007). This was also supported by a study reporting changes in D3 receptor binding, without changes in expression, in the brains of developmentally treated mice (Mizuo et al., 2004b) and by in vitro studies (Miyatake et al., 2006). The most recent studies from this group examine the development of dopaminergic neurons (Miyagawa et al., 2007b).

Most of the studies from this group, as outlined in Table 15, used prenatal/postnatal exposure. However, one study demonstrated effects with exposure only during organogenesis (Narita et al., 2007). None of the studies provided information on litter distribution or litter-based statistics.

A study from a different research group looked only at neurons that synthesize dopamine (Tando et al., 2007). This study was based on the observation that BPA binds to the γ-noradrenergic receptor (Nadal et al., 2000), a receptor for the monoamine neurotransmitters epinephrine, norepinephrine, and dopamine. The authors explored the populations of dopaminergic neurons in the substantia nigra of mice exposed developmentally to BPA. Specifically, they counted the number of neurons immunoreactive for tyrosine hydroxylase, a key enzyme in catecholamine synthesis. In addition, the distribution of cortical neurons expressing calcium-binding proteins was studied because ERβ colocalizes with these proteins. A prenatal/postnatal exposure was used. There were fewer dopaminergic neurons in substantia nigra of BPA-treated mice than controls, although the difference was significant only in females. The distribution of calcium binding proteins that colocalize with ERβ and dopamine receptors was not affected.

Effects of BPA on dopamine systems are important because dopamine regulates brainstem reward systems and motor systems.

Studies screening with a behavioral test battery: These two studies were not based on any particular hypothesis concerning BPA effects on the brain or development. They used behavioral test batteries to more generally assess potential developmental neurobehavioral toxicity. These studies used prenatal/postnatal exposures.

A laboratory that had previously studied BPA effects on dopamine systems and drugs of abuse (Suzuki et al., 2003; Narita et al., 2006, 2007) undertook a broader evaluation of behavior using the C57Bl/6 mouse (Miyagawa et al., 2007a). As in previous studies, only male mice were tested. The test battery included elevated plus maze, rotarod, light–dark box, and passive avoidance. In this study the number of dams per group was stated ($n = 10$) and the authors indicated that the pups from each litter were distributed across the 4 tests with 5–11 male offspring per group per test. There were three groups, control and two BPA doses previously studied (30 ng/g and 2 mg/g diet, corresponding to 3 μg/kg/body weight and 200 mg/kg body weight). The only treatment effect identified by statistical analysis was on the retention of the passive avoidance response as assessed by time to enter 48 hr after training. This was lower in both treated groups. A previous study (Kubo et al., 2001) used the passive avoidance test and found a lack of sex differentiation after developmental exposures to a 1.5 mg/kg d dose.

A routine guideline-style multigeneration study (Ema et al., 2001) in SD rats also evaluated reflex development prior to weaning, and exploratory activity and learning and memory in a water maze during puberty (5–7 weeks of age). Remarkably, gastric intubation was used for this long-term dosing regimen. For reflex development, males in the F_2 generation were delayed in acquisition of the negative geotaxis reflex at 0.2, 2, and 20 μg/kg d dose, and both male and female F_1 pups had earlier onset of air righting at 20 μg/kg d. There were no BPA effects on the pubertal tests. Litter-based statistics were not discussed for the behavioral tests. As in other studies in this dose range, no effects on fertility, pregnancy outcome, or postnatal mortality and weight gain were recorded.

DISCUSSION

The literature on BPA developmental toxicity contains both commercial safety-testing studies and investigator-initiated research. The safety-testing studies follow the guidelines of regulatory agencies, focusing on fetal malformation, growth, and survival, but also including postnatal reproductive system endpoints (AGD, puberty onset, estrous cycles, and sperm parameters). Investigator-initiated research has been conducted within the framework of the endocrine disruption concept, which emphasizes action at nuclear steroid hormone receptors, low dose exposures, and sexual differentiation, particularly of the nervous system. Despite a somewhat uncomfortable intersection between these two

approaches, useful integration of the experimental findings promises to further understanding of BPA as a developmental toxicant (Beronius et al., 2010; Soto et al., 2009).

Key findings concerning developmental toxicity endpoints that have appeared across the range of studies within and between laboratories include:

- Effects on offspring viability in the higher range of doses tested.
- Effects on sex-differentiation of exploratory and affective behavior at lower doses.
- Effects on immune hyperresponsiveness at lower doses.
- Effects on gender-differentiated morphology.

Of the variety of biological actions of BPA, direct regulation of gene expression in the embryo, action at membrane estrogen receptor sites, and modulation of second messenger systems are most often discussed as relevant to developmental toxicity endpoints reviewed here. Connection between a BPA biological action and an affected endpoint may be supported by similarity to a ''positive control,'' by correlations between gene expression or biochemical changes and the affected endpoint, or by targeting of the putative underlying mechanisms by drug administration or genetic manipulations. However, it is to be anticipated that multiple complex pathways mediate BPA effects on the embryo and fetus, some unique to early development, and that full description of mechanism may be quite specific to dose and period of treatment.

The large number of studies considered here have intended to provide information relevant to human health issues including:

- Hazard identification for human health risk assessment.
- Low dose endocrine disruption.
- Effects on gene expression pathways critical for embryonic and fetal development.
- Early induction of metabolic disorders like obesity.
- Developmental influences that predispose to allergy.
- Alteration in social and affective behaviors known to be shaped by hormones during development.
- Abnormalities of genital differentiation, such as hypospadia.
- Effects on dopamine systems that underlie drug abuse.

The links between the current literature, largely generated in rats and mice, and these human health issues will depend on further elucidation of mechanism and the emergence of data from human populations.

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