DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

Date: May 28, 2008

From: Division of Food Contact Notifications (DFCN)
Michelle L. Twaroski, Ph.D. (HFS-275)

Subject: Acceptance of updated reviews of the developmental neurotoxicity potential performed by Oak Ridge National Laboratory (ORNL, FDA Interagency Agreement #224-00-2615, Task #2007-20) and by Drs. Sherry A. Ferguson and Merle G. Paule at FDA's National Center for Toxicological Research

To: Food Master File (FMF) 580 – Administrative Record

As part of the updated assessment of the use of bisphenol A (BPA, CAS RN 80-05-7) in food contact materials, the Office of Food Additive Safety has completed a review of the neural and behavioral developmental toxicity of BPA. This review includes an assessment of several publications in the peer reviewed literature specific to the endpoints concluded to be of some concern in the National Toxicology Program's draft Brief on BPA[1].

The first part of the review, Attachment 1, was completed by Oak Ridge National Laboratory (ORNL, FDA Interagency Agreement #224-00-2615, Task #2007-20). ORNL primary reviewers included Drs. Carol S. Wood, Jennifer L. Rayner, and Thomas J. Sobotka. The secondary review of this report was completed by Drs. Sherry A. Ferguson and Merle G. Paule at FDA's National Center for Toxicological Research (NCTR; HFT-132).

ORNL's review consisted of two tasks. The first task was to conduct a review, which included an audit, of a literature review entitled *Literature Review of Neurobehavioral Effects of Bisphenol A*[2], previously submitted to FDA by Steven G. Hentges, Ph.D. representing the American Plastics Council. The second task was to update the review by performing a literature search, to provide a review/assessment of the literature and a weight of evidence analysis with an executive summary.

The FDA/NCTR secondary reviewers agree in general with the summaries and conclusions presented throughout the report entitled "Updated Review of Developmental Neurotoxicity Potential of Bisphenol A (BPA)". The general "weight of evidence" in establishing a no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) appears to suggest that developmental BPA treatment can cause alterations in brain development and behavior; however, the limitations noted for each particular study ranged from mild to severe. The majority of the studies appeared focused on hypothesis testing,

[1] Dated April 14, 2008; accessible at http://cerhr.niehs.nih.gov/chemicals/bisphenol/BPADraftBriefVF_04_14_08.pdf.
[2] Prepared by Exponent, 1010 14th Street, San Francisco, California, dated June 10, 2005; Attachment 2.

rather than safety assessment. With that in mind, it is difficult, if not impossible, to establish a NOAEL or LOAEL at this time.

In reviewing the ONRL updated document in comparison to the NTP report, it was noted that the search strategy omitted several papers that were pre-2004 in which the focal area was not behavioral in nature, but neurodevelopmental. In addition, an updated literature search was performed in TOXNET with PubMED and Web of Science using the term combinations with BPA, tox, neuron, develop, and brain. As part of the FDA Task Force on BPA, the OFAS requested the same NCTR reviewers perform a primary review of the publications identified either pre-2004 or in the updated literature search. Their assessment "Updated review of the developmental neurotoxicity potential of Bisphenol A (BPA)" is included as Attachment 3. This reviewer has secondary reviewed the assessment and considers it acceptable to the record.

Noteworthy, the reviewers have provided comments on the use of water extracted from polycarbonate bottles in an *in vitro* experiment conducted by Lee et al., 2008. Chemistry (HFS-275) has been provided this study to determine the relevancy of the migration experiments with regard to FDA's estimates of exposure.

Michelle L. Twaroski, Ph.D. (HFS-275)

INT: S.A. Ferguson, Ph.D. 05/28/2008
INT: M.G. Paule, Ph.D. 05/28/2008
INT: F.L. Lin, Ph.D. 05/28/2008

Attachment 1: ORNL Updated Review of Developmental Neurotoxicity Potential of Bisphenol A (BPA)
Attachment 2: Literature Review of Neurobehavioral Effects of Bisphenol A, prepared by Exponent, 1010 14th Street, San Francisco, California, dated June 10, 2005.
Attachment 3: Updated Review of the Developmental Neurotoxicity Potential of Bisphenol A (BPA)

UPDATED REVIEW OF DEVELOPMENTAL NEUROTOXICITY POTENTIAL OF BISPHENOL A (BPA)

Prepared for

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
College Park, MD 20740-3835

Prepared by

Toxicology and Hazard Assessment
Environmental Sciences Division
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Oak Ridge, TN 37831
Assignment #: 2007-20

Primary Reviewers:
Carol S. Wood, Ph.D., D.A.B.T. Signature: [redacted] Date: 5-16-08

Jennifer L. Rayner, Ph.D. Signature: [redacted] Date: 5-16-08

Thomas J. Sobotka, Ph.D. Signature: [redacted] Date: 5-16-08

Secondary Reviewer:
Robert H. Ross, M.S. Signature: [redacted] Date: 5-16-08

Quality Assurance:
Lee Ann Wilson, M.A. Signature: [redacted] Date: 5-16-08

Disclaimer

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UPDATED REVIEW OF DEVELOPMENTAL NEUROTOXICITY POTENTIAL OF BISPHENOL A (BPA)

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1. **Task 1**: Review of report titled "Exponent: Literature Review of Neurobehavioral Effects of Bisphenol A."

A summary integrating the conclusions of this review and the *ORNL* updated review can be found in Appendix A.

1.1 Summary and conclusions:

The reviewer agrees with the summaries and conclusions presented throughout the document. The information presented in the tables and appendix is correct with the exception of those noted below.

1.2 QC findings:

After review of the document, the following errors were located.

1. Page 42 Sato 2004: 3-min Openfield Activity occurred on PND 40. PND 60 is listed.
2. Page I -29 Sato 2004: Overall assessment- "BPA decreased grooming and defecation during…" should be replaced with "BPA increased grooming and decreased defecation during…"

1.3 References reviewed for Task 1:

The following references from the report were reviewed.

1. Adriani et al. 2003
2. Aloisi et al. 2002
3. Carr et al. 2003
4. Dessi-Fulgheri et al. 2002
5. EU Summary Report 2003
6. Farabollini et al. 2002
7. Gray et al. 2004
8. Ishido et al. 2004
9. Kawai et al. 2003
10. Kubo et al. 2001
11. Negishi et al. 2003
12. Negishi et al. 2004
13. Palanza et al. 2002
14. Sashihara et al. 2001
15. Sato et al. 2001
16. Schantz et al. 2001
17. Suzuki et al. 2003
18. Tyl et al. 2002

2. **Task 2**: Updated literature review of developmental neurotoxicity effects of bisphenol A (BPA)

A summary integrating the conclusions of this review and the *Exponent* review can be found in Appendix A. Additional critical analyses of each study described below, including the relevance of the findings to humans and the utility of the study for food additive regulatory decisions, can be found in Appendix B.

Search strategy:

Electronic databases searched included NTIS, HSDB, TOXLINE, PUBMED, and Web of Science. Each search was based on the chemical name and CAS number using the key terms "neuro*" and "develop*" and was limited by date from 2004 to present. Relevant articles were identified by title and abstract information, when available, and ordered. The NTP and Exponent reports were cross-referenced for additional articles that may have been missed in the searches. A number of articles from the NTP report had been sent by FDA; most were also identified in the search results. Preference was given to studies administering BPA by the oral route. Studies for which only an abstract was available were not reviewed. Studies which used cell culture or isolated tissue preparations were not included.

2.2 Review of studies identified:

2.2.1 Studies with direct treatment of animals followed by behavioral testing or biochemical measurements

2.2.1.1 Oral administration

Della Seta et al. (2005): Pregnant Sprague-Dawley rats were orally administered 40 µg/kg/day of BPA or the peanut oil vehicle using a micropipette beginning on the day after mating and continuing throughout gestation and lactation, inclusive (42 days). The rats had been trained to drink the oil from the micropipette. After delivery, maternal behavior was monitored on days 3-4 and 8-9. Thirty minutes before testing, all pups were removed from the cage. To start the test, four pups of the same sex were introduced into the dam's cage opposite the nest. The frequency, duration, and latency of elements of maternal behavior were recorded for thirty minutes. No differences from controls were noted for retrieving pups, overall lactation, being on the nest, or nest building activity.

BPA treated dams did have a significantly reduced duration of licking-grooming ($p<0.05$) and marginal reductions in frequency of licking-grooming ($p<0.09$), frequency of ano-genital licking ($p<0.08$), and duration of arched-back posture for nursing ($p<0.07$). Effects were similar for both male and female pups (see Text Table 1).

Text Table 1: Effects of BPA exposure on maternal behavior towards offspring				
	Male offspring		Female offspring	
	control	BPA	control	BPA
Days 3-4				
Ano-genital licking (f)	9.7 ± 2.3	7.6 ± 1.7	6.7 ± 1.1	4.1 ± 1.5
Ano-genital licking (d)	127.7 ± 27.1	141.7 ± 48.0	88.2 ± 18.0	57.7 ± 21.6
Licking-grooming (f)	13.9 ± 2.5	9.6 ± 1.4	11.9 ± 2.0	8.0 ± 2.2
Licking-grooming (d)	54.3 ± 9.2	32.1 ± 8.4	57.7 ± 12.8	32.6 ± 13.4
Arched-back posture (f)	3.0 ± 1.5	3.3 ± 1.0	3.0 ± 0.9	3.3 ± 1.4
Arched-back posture (d)	278.6 ± 104.9	164.6 ± 35.8	309.1 ± 102.6	175.9 ± 83.0
Days 8-9				
Ano-genital licking (f)	7.8 ± 1.7	6.7 ± 1.1	10.0 ± 0.7	7.9 ± 1.5
Ano-genital licking (d)	109.7 ± 20.1	84.4 ± 15.2	73.1 ± 7.3	81.7 ± 16.3
Licking-grooming (f)	20.7 ± 3.7	15.0 ± 3.3	24.9 ± 2.7	21.6 ± 3.7
Licking-grooming (d)	101.3 ± 28.0	50.3 ± 12.9	120.6 ± 24.0	94.9 ± 14.2
Arched-back posture (f)	2.0 ± 0.4	1.6 ± 0.6	1.7 ± 0.6	1.6 ± 0.9
Arched-back posture (d)	313.4 ± 81.9	184.3 ± 105.5	324.9 ± 140.6	59.1 ± 34.7

Data from Della Seta et al. (2005), Table 1.
f = frequency; d = duration

The LOAEL is 40 µg/kg/day based on changes in maternal behavior. A NOAEL could not be identified as only one dose of BPA was used in this study.

Della Seta et al. (2006): Male Sprague-Dawley rats (n = 7-10) were orally administered 40 µg/kg of BPA or the peanut oil vehicle using a micropipette on PND 23-30, inclusive. A concurrent positive control group was treated with 0.4 µg ethinyl estradiol (EE)/kg/day. The rats had been trained to drink the oil from the micropipette. On PND 45, animals were tested for social and non-social behavior to an object placed in the cage (4 animals/cage) and on PND >90 they were tested for sexual behavior. Animals not used for behavioral testing were sacrificed on PND 37 or 105 and blood collected for hormone determination. Body weight was recorded every two days.

Body weight was not affected by treatment. In juvenile animals (PND 45), significantly lower frequencies ($p = 0.01$) of the behaviors grouped under elements directed to the object placed in the cage (biting, sniffing, climbing) were found in animals treated with BPA and EE. Sexual behavior was clearly affected in animals treated with EE as noted by increased frequency of intromission, decreased latencies for mount and intromission, decreased duration of genital sniff, and an increase in the refractory period. A similar trend for most endpoints was found in BPA treated animals with statistical significance attained only for intromission latency. Plasma testosterone levels in the BPA treated animals were significantly lower than the control and EE treated animals at PND 37 and 105 (see Text Table 2). No differences in plasma estradiol levels were found between groups at any timepoint.

Text Table 2: Plasma Testosterone levels in male rats (ng/mL)			
Age	Oil control	BPA-treated	EE-treated
PND 37	0.49 ± 0.04	0.33[#§] ± 0.02	0.41[#] ± 0.02
PND 105	4.13 ± 0.48	1.60[#§] ± 0.41	3.50 ± 0.78

Data from Della Seta et al. (2006), Table 3.
[#]Significantly different from control.
[§]Significantly different from EE.

The LOAEL is 40 µg/kg/day based on changes in social, non-social, and sexual behavior and decreased testosterone levels. A NOAEL could not be identified as only one dose of BPA was used in this study.

Ceccarelli et al. (2007): Male and female Sprague-Dawley rats (n = 14/sex) were orally administered 40 µg BPA/kg or the peanut oil vehicle using a micropipette on PND 23-30, inclusive. A concurrent positive control group was treated with 0.4 µg ethinyl estradiol (EE)/kg/day. The rats had been trained to drink the oil from the micropipette. Half of the animals were sacrificed on PND 37 and the remainder on PND 90. At sacrifice, blood was collected for hormone assays and the animals were perfusion fixed. Coronal sections of the brain were incubated with estrogen receptor (ER)-α rabbit polyclonal antibody. ER-α immunoreactive cells were counted in selected hypothalamic areas including the arcuate nucleus, ventromedial nucleus, and medial preoptic area.

On PND 37, an increased number of ER-α labeled cells was observed in the arcuate nucleus from BPA treated males and females and in the ventromedial nucleus of females compared to the controls. On PND 90, BPA treated females had a higher number of labeled cells in the medial preoptic area than the treated males but not compared to the female controls. Plasma testosterone levels were significantly decreased in BPA treated males on PND 37; no other treatment-related differences in hormone levels were found (data presented graphically).

The LOAEL is 40 µg/kg/day based on an increased number of ER- α labeled cells and decreased testosterone levels. A NOAEL could not be identified as only one dose of BPA was used in this study.

Ishido et al. (2007): Male Wistar rats were gavaged with 600 µg BPA/pup daily from 5 days to 3 weeks of age. Doses were reported as equivalent to 12-60 mg/kg/day. Controls received the olive oil vehicle. At 4-5 weeks of age, spontaneous motor activity was measured at 15-minute intervals for 22-24 hours under a 12-hour light/dark cycle. Rats were sacrificed at 7 weeks of age and the brain processed for immunohistochemistry against tyrosine hydroxylase and in situ transferase mediated dUTP nick end-labeling (as a measure of apoptosis). Finally, the level of gene expression of the dopamine transporter was measured via RT-PCR.

Body weight was not affected by treatment. BPA treated rats had a 1.3x increase in total spontaneous motor activity during the dark cycle compared to the controls. No difference in activity level was found during the light cycle. Tyrosine hydroxylase immunoreactivity was reduced in the substantia nigra of BPA treated rats and correlated

with an increase in cells detected with nuclear condensation indicative of apoptosis. Gene expression of the dopamine transporter was completely inhibited in treated animals. (note: these results were not quantitated).

The LOAEL is 600 µg/pup/day based on increased motor activity, decreased tyrosine hydroxylase immunoreactivity, and altered gene expression. A NOAEL could not be identified as only one dose of BPA was used in this study.

Razzoli et al. (2005): Social and non-social (exploratory) behavior in adult female Mongolian gerbils, a monogamous species was examined. Pairs were established by simultaneously introducing a male and female into a cage. Beginning on the day of pairing and continuing until day 21 of cohabitation, females (n = 12/group) were orally administered 2 or 20 µg BPA/kg/day or the corn oil vehicle using a modified syringe. A concurrent positive control group was treated with 0.04 µg ethinyl estradiol/kg/day. The animals had been trained to drink the oil from the syringe. Social interactions occurring within each pair were recorded daily. After the 21 days of treatment, females were tested in a free exploratory test.

Body weight was not affected by treatment. The frequency of social sniffing was significantly increased (by 60%) in the low-dose BPA group and the estradiol group compared with the controls. No other differences were found in social behavior. In the exploratory test, significant effects included decreased time in the central unfamiliar area in the low- (60%) and high-dose (44%) BPA groups, fewer transitions to the unfamiliar area in the low- (60%) and high-dose (50%) BPA groups, fewer transitions to the home cage (29%) in the high-dose group, and less maximum time in the unfamiliar area by the low-dose (46%) group. [Note: data were presented graphically; percentages reported in NTP (2007) and verified by current reviewer].

The LOAEL was 2 µg/kg/day based on changes in social and non-social behavior and the NOAEL could not be identified.

2.2.1.2 Parenteral administration

Several studies were found in which adult or neonatal rats were administered BPA by a route other than oral, and tested for brain structure or gene expression. These mechanistic studies confirm many of the results following oral exposure and are only briefly summarized here.

MacLusky et al. (2005): Adult ovariectomized Sprague-Dawley rats (250-300 g) were used to investigate the effects of BPA on estrogen-induced formation of dendritic spine synapses on pyramidal neurons. One week after ovariectomy, animals (n = 3) were administered 60 µg estradiol/kg by subcutaneous injection and simultaneously injected with 0, 40, 120, or 400 µg BPA/kg. In another experiment, animals were injected with either 45 µg estradiol/kg, 300 µg BPA/kg, or a combination. The vehicle was sesame oil. Thirty minutes after treatment, the animals were sacrificed and the brain processed for electron microscopy of the pyramidal neurons of the hippocampus.

The formation of dendritic spine synapses induced by estradiol was inhibited by coadministration of BPA in a dose-related manner (data presented graphically). Statistical significance was attained for all BPA doses.

Uterotrophic responses were assessed in a separate assay. Rats (n = 3) were injected with either 60 µg estradiol/kg, 400 µg BPA/kg, or a combination daily for three days. Six hours after the last injection, animals were sacrificed and the uterus was removed and weighed. The uterotrophic effect of estradiol was only slightly, but not significantly, inhibited in the presence of BPA (results presented graphically).

The LOAEL was 40 µg BPA/kg based on inhibition of estradiol-induced formation of dendritic spine synapses in pyramidal neurons. A NOAEL was not identified.

Patisaul et al. (2006; 2007): Male and female Sprague-Dawley neonates were given 4 subcutaneous injections of 250 µg BPA at 12 hour intervals on PND 1 and 2. In males sacrificed on PND 19, the number of tyrosine hydroxylase immunoreactive neurons in the anteroventral periventricular nucleus of the hypothalamus (AVPV) was increased to a level similar to that of control females (i.e., demasculinization). On PND 85, another set of males was gonadectomized followed by sequential treatment with estrogen and progesterone; the number of calbindin neurons in the sexually dimorphic nucleus of the preoptic area was significantly increased. In females sacrificed on PND 19, the number of tyrosine hydroxylase immunoreactive neurons in the AVPV was significantly decreased (i.e., defeminized).

Masuo et al. (2004a,b); Ishido et al. (2005): In a series of studies, 5-day old male Wistar rats were administered 87 nmol of BPA by intracisternal injection (no approximation of this dose in mg/kg was given by the authors). As adults, the animals displayed hyperactivity and altered expression of several classes of genes in the brain.

Shikimi et al. (2004): Male and female Fisher rats were administered 50 or 500 µg BPA by daily injection into the cerebral spinal fluid on PNDs 6-9 followed by sacrifice on PND 10. The high-dose pups showed a stimulatory effect on Purkinje dendritic growth as measured by dendritic length.

2.2.2 Studies with maternal treatment followed by behavioral testing of offspring

2.2.2.1 Oral administration

Gioiosa et al. (2007): Pregnant CD-1 mice were orally administered 10 µg/kg/day of BPA or the tocopherol-stripped corn oil vehicle using a micropipette beginning on GD 11 and continuing through PND 8. The mice had been trained to drink the oil from the micropipette. Offspring were subjected to behavioral testing at 30 days of age in the novelty-seeking test and at 70 days of age in the free-exploratory open-field and elevated plus maze tests. In all tests in both prepubertal and adult offspring, BPA exposure eliminated sex-related behavioral differences observed with control animals. Generally,

the lack of sex-related differences was due to the fact that the behavior of the treated females was more similar to that of control males than to that of control females. Thus, characteristic differences between male and female mice in non-reproductive behaviors were not observed following prior exposure to BPA.

The LOAEL is 10 µg/kg/day based on elimination of sex-related behavioral differences. A NOAEL could not be identified as only one dose of BPA was used in this study.

Laviola et al. (2005): Pregnant CD-1 mice were orally administered 10 µg/kg/day of BPA or the tocopherol-stripped corn oil vehicle using a micropipette on GDs 11-18. The mice had been trained to drink the oil from the micropipette. At 60 days of age, offspring were subjected to behavioral testing which consisted of changes in the reinforcing effects of amphetamine (0, 1, or 2 mg/kg, i.p.) using the conditioned place preference paradigm. The expected dose-dependent increase in locomotor activity was observed in both sexes following amphetamine administration. Prenatal exposure to BPA did not affect the initial response to amphetamine. The conditioned response to amphetamine was not affected in males by BPA exposure. In contrast, females failed to show the conditioned response to the rewarding property of amphetamine following prenatal exposure to BPA.

The LOAEL is 10 µg/kg/day based on lack of conditioned response to amphetamine in females. A NOAEL could not be identified as only one dose of BPA was used in this study.

Mizuo et al. (2004): Female ddY mice were administered BPA in the diet at concentrations of 0, 2, 500, or 2000 mg/kg diet from mating to weaning of their pups. [Doses were not calculated by the study authors; doses are estimated to be 0.4, 100, or 400 mg/kg body wt/day using the assumption that a female mouse eats approximately 0.2 kg feed/kg body wt/day.] Male offspring were tested for place conditioning (n = 6-10) and motor activity (n = 9-10) in response to morphine (1 mg/kg, s.c.). At sacrifice guanosine-5'-diphosphate binding and expression of µ-opioid receptor mRNA were measured in three brain samples per group. Age of the animals at testing and sacrifice was not given; in other work by these authors, testing was done when offspring were 7-9 weeks of age (see next two summaries).

A dose-related increase in time spent in the compartment associated with morphine was observed with statistical significance attained at the mid- and high-dose of BPA. Animals from the high-dose group also had significantly increased motor activity after morphine injection compared with controls. No effects were observed on guanosine-5'-diphosphate binding or on expression of µ-opioid receptor mRNA.

The LOAEL is 500 mg/kg feed (estimated to be 100 mg/kg/d) based on enhanced reward effect and hyperlocomotion induced by morphine. The NOAEL is 2 mg/kg feed (0.4 mg/kg/d).

Narita et al. (2006): Female ddY mice were administered BPA in the diet at concentrations of 0, 0.03, 0.3, 3, 500, or 2000 mg/kg diet from mating to weaning of their

pups. [NTP (2007) estimated doses to be 0.006, 0.06, 0.6, 100, or 400 mg/kg body wt/day using the assumption that a female mouse eats approximately 0.2 kg feed/kg body wt/day.] At 7 weeks of age, male offspring were tested for place conditioning response and motor activity in response to morphine (1 mg/kg, s.c.). Males from the low-dose (0.03 mg/kg feed) and two highest dose (500 and 2000 mg/kg feed) groups spent significantly more time in the section of the cage associated with morphine. Total motor activity, measured in the 0.03, 3.0, and 2000 mg/kg feed groups, was significantly increased following morphine injection in the low- and high-dose groups compared with the controls. Binding of ^{35}S-guanosine-5'[γ-thio]-triphosphate in the limbic system was measured in male offspring from dams in the 0.03, 3.0, and 2000 mg/kg feed groups. Dopamine-induced binding was significantly increased at all dose levels.

Note: this paper is poorly written and difficult to interpret. The LOAEL is 0.03 mg/kg feed (estimated at 0.006 mg/kg body wt/day) based on potentiation of central dopamine receptor-dependent neurotransmission. The NOAEL was not identified.

Narita et al. (2007): In a nearly identical study to the two described above, female ddY mice were administered BPA in the diet at concentrations of 0 or 2000 mg/kg diet (approximately 400 mg/kg body wt). Treated diets were administered during implantation (GDs 0-7), organogenesis (GDs 7-14), parturition (GDs 14-20), or lactation (PNDs 0-20). At 7-9 weeks of age, male offspring were tested for place conditioning and motor activity in response to morphine and binding of ^{35}S-guanosine-5'[γ-thio]-triphosphate in the limbic system was measured. Both responses to morphine as well as receptor binding were enhanced by BPA exposure during organogenesis and lactation, but not during implantation and parturition.

The LOAEL is estimated at 400 mg/kg/day based on enhanced response to morphine following maternal BPA exposure on GD 7-14 and PNDs 0-20. A NOAEL could not be identified as only one dose of BPA was used in this study.

Ryan (2005); Ryan and Vandenbergh (2006) [mouse studies]: A series of studies was conducted to determine the effects of perinatal and lactational exposure to BPA on the onset of puberty, short-term spatial memory, and anxiety. Pregnant C57/Bl-6 mice were administered 2 or 200 µg BPA/kg/day from GD 3 through PND 21. A positive control group received 5 µg ethinyl estradiol/kg/day and the negative control received the tocopheral-stripped corn oil vehicle. The dose was administered to the back of the throat. One week after weaning on PND 21, female offspring were ovariectomized; after a two week recovery period, each animal was subjected to behavioral testing. The onset of puberty was monitored in non-ovariectomized animals (n = 14); the first day of puberty was defined as the day on which cornified cells were first detected in a vaginal smear.

No effects of treatment were noted on offspring body weight, litter size, or anogenital distance. The onset of puberty was significantly earlier in the females from high-dose BPA and estradiol treated dams (approximately 4 and 6 days, respectively, earlier than in controls; data presented graphically).

Two anxiety tests were conducted, the elevated-plus maze and the light/dark preference chamber. All animals were tested in both apparatuses (n = 14). In the elevated-plus maze test, animals from the high-dose BPA group spent slightly less time in the open arms than the controls but statistical significance was not attained. Animals from the estradiol group spent significantly less time in the open arms than did the controls. In the light/dark preference chamber, animals from both the high-dose BPA and estradiol groups spent significantly less time in the lighted section than the controls. The authors stated that the results were consistent with an increased level of anxiety.

Short-term spatial memory was assessed by the radial-arm maze and the Barnes maze. Each animal was tested in both assays (n = 16). Overall performance in both mazes by animals from the BPA groups did not differ significantly from that of the controls. The estradiol treated animals had significantly fewer errors in both mazes than did the controls.

The LOAEL for adult female mice following peri- and post-natal exposure is 200 µg/kg/day based on early onset of puberty and increased anxiety. The NOAEL is 2 µg/kg/day.

Ryan (2005); Ryan et al. (2006) [rat studies]: A series of studies was conducted to determine the effects of perinatal and lactational exposure to BPA on the onset of puberty, saccharine preference, and motor activity. Pregnant Long-Evans rats were administered 2, 20, or 200 µg BPA/kg/day from GD 7 through PND 18 by oral gavage. Positive control groups received 0.05, 0.5, 5, or 50 µg ethinyl estradiol/kg/day and the negative control received the corn oil vehicle. Offspring were weaned on PND 23 and only females were used for further testing. Female offspring were at least 50 days old before further behavioral testing.

Maternal treatment with BPA did not affect number of implantations, pup mortality, or day of vaginal opening. The high-dose estradiol group had a decreased number of implantations resulting in a reduced number of pups per litter. Vaginal opening was significantly earlier (about 2 days; data presented graphically) in the 5 µg ethinyl estradiol/kg/day group than the controls (the 50 µg ethinyl estradiol/kg/day group was not observed for vaginal opening).

As adults, intact females were tested for saccharine preference. No clear effect of prior BPA exposure from maternal treatment was seen; the mid-dose group showed a significant decrease in the amount of saccharine consumed on two of the five testing days compared to the controls. No dose-response was evident as the high-dose group was similar to the controls. With animals from the estradiol treated groups, a dose-related decrease in saccharine preference was observed at >5 µg ethinyl estradiol/kg/day.

Prior to motor activity testing, adult females were ovariectomized and allowed two weeks to recover. One-half of the animals were then administered 275 µg ethinyl estradiol/kg/day for 14 days. Motor activity was assessed in a figure 8 maze in which animals were allowed to freely explore for 10 hours. Increased motor activity was

observed in the low-dose BPA and high-dose estradiol groups in the absence of supplemental estradiol. No differences were found between groups when animals were supplemented with estradiol for 14 days. However, when the data were analyzed as the difference in activity before and after supplemental estradiol, all groups previously exposed to BPA had lower activity than the controls. The author attributed this to a decrease in sensitivity to estradiol following perinatal and lactational exposure to BPA. It was noted that the motor activity data were highly variable between individuals making interpretation somewhat difficult.

The LOAEL was 2 µg/kg/day based on decreased sensitivity to estrogen as measured by motor activity. The NOAEL was not identified.

Xu et al. (2007): Pregnant Sprague-Dawley rats (n = 8-9) were administered 0.1 or 50 mg BPA/L in the drinking water beginning on GD 11 and continuing until PND 21. Controls received tap water. Doses to the dams were not reported; estimated doses are 0.02 and 10 mg/kg/day based on the average water consumption of 0.049 L/day and assuming a body weight of 0.250 kg for a rat. Offspring were weaned on PND 21 and the males and females were subjected to behavioral tests, and specific hormone and receptor measurements. Neurobehavioral tests included open-field behavior at 6 weeks of age and Morris water maze test at 10 weeks. Free T4 levels in whole blood were measured in pups and dams on PND 0, 7, and 21 and in dams on GD 11 and 20. Protein and mRNA levels for thyroid hormone receptor α/β, RC3/neurogranin, and steroid hormone receptor coactivator-1 were quantitated in the hippocampus from low-dose male pups.

Male pups from the low-dose group showed increased motor activity and rearing in the open field and increased latency in the Morris water maze. No effects on females or high-dose males were observed. Free T4 levels were significantly decreased in low-dose dams on PNDs 0 and 7 and increased in both treated groups of male pups on PND 7 followed by a decrease on PND 21. Levels of thyroid hormone receptor α/β and RC3/neurogranin in male pups were not affected by maternal treatment. Steroid hormone receptor coactivator-1 was significantly up-regulated on PNDs 5 and 7 in low-dose male pups.

The LOAEL was 0.1 mg/L (0.02 mg/kg/day) based on increased motor activity, decreased learning/memory, and changes in thyroid hormone levels in male offspring. The NOAEL was not identified.

Fujimoto et al. (2006): Pregnant Wistar rats (n = 6) were administered 0.1 ppm BPA in the drinking water beginning on GD 13 until parturition. Controls received tap water. Dose to the dams was reported as 15 µg/kg/day. Offspring were weaned on PND 21 and the males and females (n = 20-24/sex/group) were subjected to a series of behavioral tests as adults. Neurobehavioral tests included open-field behavior at 6 weeks of age, elevated plus maze test at 7 weeks, passive avoidance at 8 weeks, and forced swimming test at 9 weeks.

In the control group, rearing frequency and duration were significantly higher in females than in males. This difference was not observed in offspring from BPA treated dams. Males from the BPA group had a significant increase in rearing duration compared to control males. In the forced swimming test, control females were reported in this study to have struggled more than control males, but this difference was not observed between the BPA males and females. For the BPA males, the duration of immobility was increased in the forced swimming test compared to the control males. No effects of treatment were found in the elevated plus maze and passive avoidance tests. The authors concluded that BPA exposure of male offspring during the last week of gestation impaired sexual differentiation of rearing and struggling behaviors.

The LOAEL is 15 µg/kg/day based on impaired sexual differentiation of rearing and struggling behaviors. A NOAEL could not be identified as only one dose of BPA was used in this study.

Porrini et al. (2005): Pregnant Sprague-Dawley rats were orally administered 40 µg/kg/day of BPA or the peanut oil vehicle using a micropipette beginning on the day after mating and continuing throughout gestation and lactation. The rats had been trained to drink the oil from the micropipette. Pups were weaned on PND 21 and behavioral observations of the females were conducted on PND 35, 45, and 55. The components of behaviors were divided into six factors: social and non-social exploration, defensive toward males, play with males, play with females, low-intensity mating behavior, and social grooming.

Female offspring born to dams treated with BPA had a significant increase in exploration at 35 and 45 days, and significant decreases in play with males and social grooming at 45 days.

The LOAEL is 40 µg/kg/day based on increased exploration and decreases in play and social behaviors in females. A NOAEL could not be identified as only one dose of BPA was used in this study.

Negishi et al. (2004): Pregnant F344/N rats were administered 0.1 mg BPA/kg/day by oral gavage beginning on GD 3 until PND 20. Controls received the corn oil vehicle. Offspring were weaned on PND 21 and the males (n = 8-10/group) were subjected to a series of behavioral tests as adults. Female offspring were not tested. Neurobehavioral tests included open-field behavior at 8 weeks of age, spontaneous motor activity at 12 weeks, passive avoidance at 13 weeks, elevated plus-maze test at 14 weeks, and active avoidance at 15 weeks. At 22-24 weeks of age, the males underwent a monoamine-disruption test by injection with *trans*-2-phenylcyclopropyl amine hydrochloride followed by measurement of spontaneous activity and open-field behavior.

Maternal and male offspring body weight and organ weight and litter parameters were not affected by treatment. For BPA-exposed male offspring, results of open-field, spontaneous motor activity, and elevated plus-maze tests were similar to the controls. In the passive avoidance test during the retention trial, the BPA group showed significant

hesitation (increased latency) to enter the dark compartment. In the active avoidance test, the treated group had significantly fewer avoidance responses during the first, second, and third (of 5) sessions compared with the controls. The frequency of failure of avoidance was significantly higher in the BPA group. BPA treated animals failed to show an increase in motor activity in response to *trans*-2-phenylcyclopropyl amine hydrochloride. Results were interpreted by the study authors to indicate that BPA exposure to dams during gestation and lactation irreversibly affected perception of fear-provoking stimuli and monoaminergic neural pathways in male offspring.

The LOAEL is 0.1 mg/kg/day based on altered perception of fear-provoking stimuli. A NOAEL could not be identified as only one dose of BPA was used in this study.

2.2.2.2 Parenteral administration

Several studies were found in which pregnant mice were administered BPA by a route other than oral, and the offspring were tested for neurobehavioral alterations and brain structure or gene expression. These mechanistic studies confirm many of the results following oral exposure and are only briefly summarized here.

Rubin et al. (2006): Sex differences in open field activity seen in controls were not observed in 6-9-week old male and female mouse offspring from dams administered 250 ng/kg/day via osmotic pump from GD 8 through PND 16. The number of tyrosine hydroxylase neurons was decreased in female offspring following a maternal dose of 25 ng/kg/day.

Nakamura et al. (2006; 2007): Gene expression and cellular architecture were altered in the cortex of mice offspring following subcutaneous injection of 20 µg BPA/kg/day to pregnant animals daily during gestation. Some of the structural changes persisted into adulthood.

2.2.3 Studies with maternal treatment followed by biochemical measurements in the offspring

2.2.3.1 Oral administration

Kawai et al. (2007): Pregnant ICR mice were orally administered 2 µg BPA/kg/day using a micropipette on GDs 11-17; controls were given the corn oil vehicle. Pups were weaned on PND 21 and males only were used for further investigation. Blood was collected (n = 10-18/group) during weeks 4-5, 8-9, and 12-13 after birth for testosterone measurement. At 5, 9, and 13 weeks of age, males were sacrificed (n = 8-12/group) and the brain processed for immunoreactivity of estrogen receptors α and β and of serotonin and the serotonin transporter.

Estrogen receptors α and β were significantly increased in BPA exposed animals at weeks 5 and 13, but not at week 9, compared with control levels. Serotonin and serotonin transporter levels from the BPA treated group were similar to control levels at all

sampling times. Testosterone levels remained fairly constant (~500 ng/dL) at all time points in the BPA group while a large increase was measured in the control group at weeks 12-13 (~1500 ng/dL) (data presented graphically).

The LOAEL is 2 µg/kg/day based on increased estrogen receptor expression and decreased testosterone at puberty. A NOAEL could not be identified as only one dose of BPA was used in this study.

Tando et al. (2007): Female ddY mice were administered BPA in the diet at concentrations of 0, 3, or 8000 mg/kg diet from mating to weaning of their pups. Doses to the dams were not calculated by the study authors. [The estimated doses are 0.6 or 1600 mg/kg body wt/day using the assumption that a female mouse eats approximately 0.2 kg feed/kg body wt/day.] Pups were weaned on PND 21. At 8-11 weeks of age, the offspring were sacrificed and the brain prepared for immunohistochemistry of calcium-binding proteins and tyrosine hydroxylase.

No differences between the treated and control groups were observed in the density of calcium-binding proteins (calbindin, calretinin, parvalbumin) in the somatosensory cortex. The number of tyrosine hydroxylase-positive neurons in the substantia nigra was significantly decreased in female offspring from low-dose dams. No treatment-related effects on tyrosine hydroxylase were found in high-dose females or in males at either dose.

The LOAEL is 3 mg/kg diet (estimated to be 0.6 mg/kg body wt/day) based on effects in female offspring. The NOAEL was not identified.

Nishizawa et al. (2005a): Pregnant ICR mice (n = 12) were orally (not otherwise specified) administered BPA at doses of 0, 0.00002, 0.002, 0.20, or 20 mg/kg/day from either 6.5-13.5 or 6.5-17.5 days post coitum. Controls received the olive oil vehicle. Dams were sacrificed on day 14.5 or 18.5 post coitum and the embryos removed and dissected to isolate cerebrum, cerebellum, ovaries, and testes. Expression of mRNA for retinoic acid, retinoid X, and arylhydrocarbon receptors was measured in fetal tissues by real-time reverse transcription-polymerase chain reaction analyses.

All three receptor mRNAs were increased in all tissues on both day 14.5 and 18.5 with the exception of retinoid X in the cerebellum on day 18.5. Brain tissues generally responded in a U-shaped dose response with the greatest increases at the low dose and two highest doses. In the gonads, retinoic acid receptor mRNA was increased in the 0.00002, 0.20, and 20 mg/kg/day groups at day 14.5 and in the 20 mg/kg/day group at day 18.5; retinoid X mRNA was increased at 20 mg/kg/day on day 14.5 and at 0.2 mg/kg/day on day 18.5; and arylhydrocarbon receptor mRNA was increased in the low- and high-dose groups at day 14.5 and in the 0.00002, 0.002, and 0.20 mg/kg/day groups at day 18.5.

The LOAEL was 0.00002 mg/kg/day based on upregulation of receptors in brain and gonads. The NOAEL was not identified.

Nishizawa et al. (2005b): Pregnant ICR mice (n = 12) were orally (not otherwise specified) administered BPA at doses of 0, 0.00002, 0.002, 0.20, or 20 mg/kg/day from either 6.5-13.5 or 6.5-17.5 days post coitum. Negative controls received the olive oil vehicle and a positive control group received 17β-estradiol. Dams were sacrificed on day 14.5 or 18.5 post coitum and the embryos removed and dissected to isolate cerebrum, cerebellum, ovaries, and testes. Levels of mRNA for arylhydrocarbon receptor (AhR), arylhydrocarbon receptor repressor (AhRR), arylhydrocarbon receptor nuclear translocator (Arnt), CYP1A1, and glutathione *S*-transferase (GST) were measured in fetal tissues by real-time reverse transcription-polymerase chain reaction analyses. Protein levels for CYP1A1 and glutathione *S*-transferase were measured in embryonic liver by Western immunoblotting.

All five mRNAs were increased in all tissues on both days 14.5 and 18.5. For AhR, AhRR, and Arnt, brain tissues generally responded in a U-shaped dose response with the greatest increases at the low dose and two highest doses. For CYP1A1 and GST, mRNA levels in brain were increased on day 18.5 at the two highest doses. In the gonads, mRNA levels for AhR, AhRR, and Arnt were upregulated at the lowest doses with the greatest effect on day 18.5. GST mRNA levels in gonads and GST and CYP1A1 protein levels in liver were increased on day 18.5.

The LOAEL was 0.00002 mg/kg/day based on upregulation of mRNA levels for AhR and related factors in brain and gonads. The NOAEL was not identified.

Honma et al. (2006): BPA was administered by oral gavage to pregnant Sprague-Dawley (n = 5-6/group) rats at doses of 0, 4, 40, or 400 mg/kg/day from GD 6 through PND 20. Controls received the corn oil vehicle. The high dose group was terminated due to excessive mortality; additional details were not given. The brain content of several neurotransmitters was assayed in female offspring at 1, 3, 6, and 9 weeks of age and in dams 3 weeks after delivery. Data from offspring were highly variable, not dose-related and difficult to interpret. No consistent effects of maternal treatment were observed in offspring at 1 week or 9 weeks of age. In 3 week old pups, 3,4-dihydroxyphenylacetic acid was increased at 40 mg/kg/day and homovanillic acid, serotonin, and 5-hydroxyindoleacetic acid were increased at 4 mg/kg/day in some brain regions. At 6 weeks of age, levels of choline were increased only at 4 mg/kg/day and only in the hippocampus and striatum. In dams, an increase in homovanillic acid levels was observed in both dose groups, but did not attain statistical significance. Dopamine was significantly increased in low-dose dams in the hippocampus.

The LOAEL is 4 mg/kg/day based on non-dose-related changes in some neurotransmitters in both dams and female offspring. The NOAEL was not identified.

Facciolo et al. (2005): Adult female Sprague-Dawley rats were orally administered 40 or 400 µg BPA/kg/day by pipette; controls received the arachis oil vehicle. Treatment began 8 days before mating and continued throughout mating, gestation, and lactation. The brain was removed and sectioned from female offspring on PND 7 or 55 to

determine the effects of BPA on expression of somatostatin subtype 3 (*sst$_3$*) receptor mRNA and whether the αGABA$_A$ receptor is involved in the effect. Dose-related reductions in *sst$_3$* mRNA were observed in some brain regions in both neonate and adult animals while enhancements occurred in other regions. Even greater upregulated and downregulated expression patterns were seen in the presence of αGABA$_A$ receptor agonists.

The LOAEL was 40 µg/kg/day based on changes in *sst$_3$* mRNA expression patterns in female offspring brain. The NOAEL was not identified.

Zoeller et al. (2005): Female Sprague-Dawley rats were administered 0, 1, 10, or 50 mg BPA/kg/day on a wafer which the rats were trained to eat. Treatment began on GD 6 and continued "throughout the experiment" which is presumed to be during gestation and lactation. Offspring were sacrificed on PNDs 4, 8, 15, and 35; blood was collected for T4 and TSH measurement in males and females and brain processed for quantitation of RC3/neurogranin mRNA levels in males.

Dams had a significant, dose-related decrease in body weight gain during gestation. Pup body weight was unaffected by treatment. Total T4 was significantly increased in male and female pups from all dose groups on PND 15. No treatment-related effects were observed on TSH levels. RC3/neurogranin expression was enhanced in the dentate gyrus from all treated males on PND 15.

The LOAEL was 1 mg/kg/day based on increased T4 and RC3/neurogranin levels and the NOAEL was not identified.

Funabashi et al. (2004): Pregnant Wistar rats were administered either 0.1% ethanol or 10 mg BPA/L in the drinking water until weaning of their offspring on PND 21. The day of treatment initiation was not specified although treatment was during gestation and lactation. The dose to the dams was estimated by the authors as 2.5 mg/kg/day. At 4-7 months of age, male and female offspring were killed for determination of the numbers of corticotrophin-releasing neurons in the preoptic area and bed nucleus of the stria terminalis via immunocytochemistry techniques. Female offspring were killed in proestrus.

In controls, the bed nucleus of the stria terminalis from females contained significantly more corticotrophin-releasing neurons than that from males. This sex difference was not observed in BPA treated animals due to both an increase in neurons in males and a decrease in females. In the preoptic area, control females also had significantly more neurons than males but BPA treatment did not affect this sex difference in this region.

The LOAEL is 2.5 mg/kg/day based on a lack of sex-related differences in the numbers of corticotrophin-releasing neurons. A NOAEL could not be identified as only one dose of BPA was used in this study.

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3. **Task 3**: Executive Summary

In the first task of this assignment, the report titled "Exponent: Literature Review of Neurobehavioral Effects of Bisphenol A" was reviewed for accuracy and confidence in the conclusions. Overall, the reviewers agree with the summaries and conclusions presented throughout the document. The information presented in the tables and appendix is correct with two minor exceptions that do not affect the conclusions drawn.

The second task in this assignment was an updated literature review of bisphenol A (BPA) since the document in Task 1 was published. The search strategy was designed to limit the findings to studies which assessed neurotoxicity or developmental neurotoxicity endpoints. Studies were found in which testing was conducted on the treated animals and in which testing was conducted on the offspring following maternal treatment. Endpoints included both behavioral and biochemical measurements. Preference was given to studies which used oral administration.

Changes in behavior were found in animals directly treated orally with BPA (Summary Table 1). Female gerbils orally administered a dose as low as 0.002 mg/kg/day displayed changes in social and non-social behavior after 21 days of treatment (Razzoli et al., 2005). Adult female rats treated orally with 0.04 mg/kg/day throughout mating, gestation, and lactation showed slight decreases in attention given to pups (Della Seta et al., 2005). This same dose administered to male pups on PNDs 23-30 resulted in changes in social, non-social, and sexual behavior as adults (Della Seta et al., 2006). Motor activity was increased in adult male rats following oral administration of 0.60 mg/animal/day on PNDs 5-21 (Ishido et al., 2007).

Behavioral changes were also found in adult offspring from dams treated orally with BPA during gestation and lactation (Summary Table 2). Studies in which dams were treated by gavage or orally by pipette are particularly important as the potential for direct exposure of the pups was eliminated. In mice, maternal treatment with 0.01 mg/kg/day resulted in changes in open field behavior and the conditioned response to amphetamine in adult female offspring (Gioiosa et al., 2007; Laviola et al., 2005). A higher maternal dose of 0.20 mg/kg/day induced early onset of puberty and increased anxiety in adult female offspring (Ryan 2005; Ryan and Vandenbergh 2006). Female rat offspring had decreased motor activity in response to estrogen following maternal treatment with 0.002 mg/kg/day (Ryan 2005; Ryan et al., 2006). Also in rats, social and non-social behavior

was altered in females following maternal treatment with 0.04 mg/kg/day (Porrini et al., 2005) and perception of fear-provoking stimuli was changed in males after maternal treatment with 0.10 mg/kg/day (Negishi et al., 2004). Similar behavioral changes, as well as differences in responses to morphine, were observed in offspring following maternal treatment via drinking water or feed; however, direct exposure of the pups can not be ruled out in these studies.

Biochemical changes in the brain were found in treated animals and in offspring from dams treated with BPA during gestation and lactation. Direct treatment of male and female rat offspring with 0.04 mg/kg/day on PNDs 23-30 resulted in decreases in testosterone levels in males as adults and changes in estrogen receptor expression in males and females (Della Seta et al., 2006; Ceccarelli et al., 2007). Tyrosine hydroxylase immunoreactivity and gene expression of the dopamine transporter were decreased in adult male rats following oral administration of 0.60 mg/animal/day on PNDs 5-21 (Ishido et al., 2007). Up-regulated and down-regulated expression patterns were found for receptors and genes in various brain regions of offspring from treated dams. Estrogen receptors were increased and testosterone levels at puberty were decreased in male offspring from dams treated with 0.002 mg/kg/day during gestation (Kawai et al., 2007) and somatostatin mRNA expression patterns were altered in various brain regions of female offspring following maternal treatment with 0.04 mg/kg/day throughout mating, gestation, and lactation. Two mechanistic studies measured upregulation of receptors in the brain of mid- and late-gestation embryos following extremely low- (0.00002 mg/kg/day) and high- (20 mg/kg/day) dose maternal treatment (Nishizawa et al., 2005a; 2005b).

Among the studies reviewed above, the overall LOAEL for animals treated directly with BPA is 0.002 mg/kg/day based on changes in social and non-social behavior of adult female Mongolian gerbils. A NOAEL was not identified because no study used a lower dose.

Among the studies reviewed above, the overall LOAEL for offspring from dams treated with BPA is 0.002 mg/kg/day based on decreased sensitivity of rats to estrogen as measured by motor activity and increased estrogen receptor expression with decreased testosterone levels in male mice. A NOAEL was not identified; although lower doses were used, the endpoints were mechanistic in nature and of unknown relevance.

SUMMARY TABLE 1: Summary of studies with testing following direct oral treatment of animals			
Species (treatment interval; method)	**LOAEL/NOAEL**	**Effects**	**Reference**
Sprague-Dawley rats (GD 1 – lactation; pipette)	LOAEL = 0.04 mg/kg/d NOAEL = none; one dose used	Altered maternal behavior	Della Seta et al. 2005
Sprague-Dawley male rat pups (PNDs 23-30; pipette)	LOAEL = 0.04 mg/kg/d NOAEL = none; one dose used	Changes in social, non-social, and sexual behavior; decreased testosterone levels	Della Seta et al. 2006
Sprague-Dawley rat pups (PNDs 23-30; pipette)	LOAEL = 0.04 mg/kg/d NOAEL = none; one dose used	Increased number of ER-α labeled cells in brain of males and females; decreased testosterone levels in males	Ceccarelli et al. 2007
Wistar male rats (PND 5 – 21; orally)	LOAEL = 0.60 mg/pup NOAEL = none; one dose used	Increased motor activity; decreased tyrosine hydroxylase immunoreactivity; altered gene expression	Ishido et al. 2007
Adult Female Mongolian gerbils (day of pairing for 21 days; orally)	LOAEL = 0.002 mg/kg/d (lowest dose tested) NOAEL = none	Changes in social and non-social behavior	Razzoli et al. 2005

SUMMARY TABLE 2: Summary of studies with oral maternal treatment followed by behavioral testing of offspring			
Species (treatment interval; method)	**LOAEL/NOAEL**	**Effects**	**Reference**
CD-1 mouse (GD 11 – PND 8; pipette)	LOAEL = 0.01 mg/kg/d NOAEL = none; one dose used	Elimination of sex-related behavioral differences	Gioiosa et al. 2007
CD-1 mouse (GD 11 – 18; pipette)	LOAEL = 0.01 mg/kg/d NOAEL = none; one dose used	Lack of conditioned response to amphetamine in females	Laviola et al. 2005
ddY mice (mating to weaning; diet)	LOAEL = 100 mg/kg/d NOAEL = 0.4 mg/kg/d	Enhanced reward effect and hyperlocomotion induced by morphine	Mizuo et al. 2004
ddY mice (mating to weaning; diet)	LOAEL = 0.006 mg/kg/d (lowest dose tested) NOAEL = none	Potentiation of central dopamine receptor-dependent neurotransmission	Narita et al. 2006
ddY mice (GDs 0-7, 7-14, 14-20; or PNDs 0-20; diet)	LOAEL = 400 mg/kg/day NOAEL = none; one dose used	Enhanced response to morphine from exposures GD 7-14 and PNDs 0-20	Narita et al. 2007
C57/Bl6 mice (GD 3 – PND 21; pipette)	LOAEL = 0.20 mg/kg/d NOAEL = 0.002 mg/kg/d	Early onset of puberty and increased anxiety in females	Ryan 2005
Long-Evans rats (GD 7 – PND 18; gavage)	LOAEL = 0.002 mg/kg/d (lowest dose tested) NOAEL = none	Decreased sensitivity to estrogen	Ryan 2005
Sprague-Dawley rats (GD 11 – PND 21; drinking water)	LOAEL = 0.02 mg/kg/d (lowest dose tested) NOAEL = none	Increased motor activity, decreased learning/memory, changes in thyroid hormone levels in males	Xu et al. 2007
Wistar rats (GD 13 – parturition; drinking water)	LOAEL = 0.015 mg/kg/d NOAEL = none; one dose used	Impaired sexual differentiation of rearing and struggling behaviors in males	Fujimoto et al. 2006
Sprague-Dawley rats (GD 1 – lactation; pipette)	LOAEL = 0.04 mg/kg/d NOAEL = none; one dose used	Increased exploration, decreases in play and social behaviors in females	Porrini et al. 2005
F344/N rats (GD 3 – PND 20; gavage)	LOAEL = 0.10 mg/kg/d NOAEL = none; one dose used	Altered perception of fear-provoking stimuli and monoaminergic neural pathways in males	Negishi et al. 2004

APPENDIX A: Integration of Reports

***Exponent* "Literature Review of Neurobehavioral Effects of Bisphenol A"**
and
***ORNL* "Updated Review of Developmental Neurotoxicity Potential of Bisphenol A (BPA)"**

Thomas J. Sobotka, Ph.D.
April 8, 2008

Exponent provided an initial document reviewing and summarizing the published literature on neurobehavioral toxicity of Bisphenol A (BPA), spanning the years 2001 through 2004. Subsequently, an updated literature review and summary of the published literature from 2005 through 2007, with several overlapping papers published in 2004, was conducted by *ORNL*. This present document is intended to provide an integrated summary of the conclusions regarding the neurotoxicity potential of BPA based on the *Exponent* and *ORNL* literature review reports. The overall purpose is to: a) characterize what is known about the neuro-behavioral toxicity of BPA, and b) provide a summary opinion on the weight of evidence concerning effects of BPA on neuro-behavioral toxicity endpoints at low doses.

BACKGROUND INTRODUCTION

The literature on the neurobehavioral effects of BPA is focused almost exclusively on the effects of developmental exposures. This emphasis is due to the fact that BPA has been shown to be weakly estrogenic, with a significantly lower potency than estradiol *(EU Summary Risk Assessment Report 2003; Gray et al. 2004).* Estrogen plays a role in the appropriate development of the brain and subsequent behavior. A predominant hypothesis regarding estrogenic effects on brain development is that the original phenotype of the brain is female in mammals, and some areas of the brain are masculinized in genetic male offspring when testosterone is converted to estradiol locally in the brain during a critical perinatal period (for reviews see *Cooke et al., 1998; De Vries and Simerly, 2002; McEwen and Alves, 1999;* and *Schantz and Widholm, 2001*).
It is hypothesized that the developing female brain is not modified by estradiol during the same period, because circulating estradiol from the dam is bound to alpha-fetoprotein *(Bakker et al, 2006).* In rodents, this critical period of sexual differentiation of the brain is believed to be a few days before birth to approximately 10 days after birth *(*for review see *Schantz and Widholm, 2001).* During this period, the rodent brain is sensitive not only to estrogens but also to direct effects of androgens on the brain. As a result of these hormonal influences, there are normal sex differences in brain structure and function. The hypotheses regarding the exact mechanism of sexual differentiation are controversial, and the link between rodents and humans is an area of active research.

Overview of the BPA Developmental Neurotoxicity Findings

Exponent reviewed 18 studies that investigated BPA's potential for neurobehavioral effects following developmental exposures. *ORNL* identified and reviewed an additional

31 developmental and 2 non-developmental studies. All of the developmental studies were conducted using rats or mice. Typically, exposure levels below the *U.S. Environmental Protection Agency's (EPA's*) lowest-observed-adverse-effect level (LOAEL) of 50 mg/kg/day were included. This LOAEL is used by EPA to calculate the RfD. Most of the developmental studies (15 *Exponent*; 22 *ORNL*) use the oral route of exposure (gavage, micropipette, diet, water), which is the most relevant route of exposure to humans and the most useful for regulatory decisions. The non-oral studies (3 *Exponent*; 9 *ORNL*) injected BPA into the experimental animals by a variety of routes (subcutaneous, intracisternal, and intracerebral). A variety of exposure regimens were used in which animals were given BPA for various intervals during gestation, lactation and/or after weaning. At least 30 oral studies (15 *Exponent*; 15 *ORNL*) exposed dams to BPA throughout gestation and/or lactation; the remaining studies, including the non-oral studies, used exposure durations of 1 to several days during select periods of gestation or lactation. The general groups of developmental neuro/behavioral toxicological endpoints identified in the studies reviewed by *Exponent* and *ORNL* include the following:

Reported Effects of BPA on Neurobehavioral and Other Indices of Developmental Neurotoxicity

Ontogeny of Sensory/Motor Behaviors and Reflexes

Commonly Measured Gross Behaviors
- **Activity and Rearing**
- **Grooming and Open-Field Defecation**

Complex/Cognitive Behaviors
- **Learning and Memory**
- **Operant Behavior for Delayed Larger Reinforcement**

Complex/Emotional Behaviors
- **Stress/Anxiety**
- **Pain-Related Behaviors/Opioid System**
- **Social Behaviors**
- **Social Play/Non-social Behaviors**
- **Aggression**
- **Socio-sexual Behavior**
- **Maternal Behavior**

Pharmacologic Challenge and Relevant Morphochemical Findings Related to Development of Dopaminergic/Monoaminergic Circuitry

Hormone Levels and Related Biochemical Factors

Factors Related to Sexual Development and Differentiation
- **Sexually Dimorphic Brain Morphology and Cytochemistry**
- **Sexually Dimorphic Behavioral Measures**

The next section summarizes the evaluation of reported effects of developmental exposure to BPA on each of the above general categories of effects. For a more detailed listing, discussion and critique of the studies and results regarding these effects refer to the original *Exponent* and *ORNL* review documents (appended). Note: studies in which intracisternal or intracerebral dosing was used were not included in the overall (weight of evidence) evaluation.

Reported Effects of BPA on Neurobehavioral and Other Indices of Developmental Neurotoxicity

Ontogeny of Sensory/Motor Behaviors and Reflexes

Two studies reviewed by *Exponent* evaluated the impact of BPA exposure on ontogeny of sensory/motor behaviors and reflexes (*Ema et al., 2001 and Palanza et al., 2002*). Both authors concluded that there were no treatment-related adverse effects of BPA on development of several behaviors. The behaviors and reflexes measured included development of grasp reflex and pivoting response, surface righting reflex, mid-air righting reflex, negative geotaxis reflex, straight-line walking, and cliff avoidance behavior. The *ORNL* updated review found no additional relevant information on the ontogeny of behavior during the preweaning period of development.

Commonly Measured Gross Behaviors

Activity, rearing, grooming, and defecation were commonly measured gross behaviors. The effects for activity and rearing, grooming, and defections are summarized, below. As indices of chemically-induced neurobehavioral toxicity, activity (when defined as horizontal movement), rearing, grooming, and defecation are related measurements that should be evaluated together in terms of a pattern of overall effect. However, in studies dealing with potential developmental effects of endocrine disrupting chemicals, activity/rearing, apart from grooming and defecation measures, may have particular use in indexing changes in sexually dimorphic behavior.

Activity and Rearing

Activity and rearing behaviors in experimental offspring were evaluated by several investigators in the studies reviewed by *Exponent* and were found to be variably affected with decreases (activity), increases (rearing) and no changes (activity and rearing) being reported and no corroboration among studies. However, several more recent studies (*ORNL* review) reported variably increased activities in offspring exposed perinatally to BPA. Adult male offspring (females not tested; *Ishido et al., 2007)* of dams exposed by gavage to an estimated dose of 12-60 mg BPA/kg/day from PND 5 – 21 showed increased activity; increased activity was reported in adult female offspring (males not tested; *Ryan, 2005*) of dams gavaged with BPA at 0.002 mg/kg/day from GD 7 – PND 21 but not at higher doses up to 0.2 mg/k/day; increased activity and rearing was found in 6 week old male, but not female, offspring (*Xu et al., 2007*) of dams given 0.02 mg/kg/day in drinking water from GD 11- PND 21, but not the higher dose of 10 mg/kg/day; and increased rearing was seen in 6 week old male, but not female,

offspring (*Fujimoto et al., 2006*) of dams given BPA in drinking water at a dose of 0.015 mg/kg/day from GD 13 until parturition. Despite the diversity of experimental designs, several studies reported relatively variable (nonmonotonic in at least two studies) increases in activity (male and female) and rearing (male) in adult experimental offspring. Confirmation of these findings using well-designed protocols should provide sufficient information to clarify the dose response relationship and to help determine the biological significance of these effects. As will be discussed below, treatment related changes in rearing behavior may also be viewed in the context of certain behaviors, such as rearing, being considered to be sexually dimorphic, that is behaviors that are specifically different between males and females.

Grooming and Open-Field Defecation

The *Exponent* review of studies concluded that there was no consistent evidence of adverse effects on self-grooming or open-field defecation in offspring following developmental exposure to BPA. The *ORNL* review provided no additional information to modify this conclusion.

Complex/Cognitive Behaviors

Learning and Memory

Exponent reviewed a number of studies that investigated the cognitive effects of perinatal BPA exposure. Citing numerous reasons, such as lack of replication of effect at two very close dose levels within the same study, inconsistent effects within the same cognitive test, treatment related improvement rather than deficient performance, performance changes confounded by prior testing, uncertainties in numbers of animals tested and whether the litter was considered the experimental unit, *Exponent* determined that that there was no consistent evidence indicating that developmental exposure to BPA causes adverse effects on offspring learning and memory at doses approximating 10^{-1} mg/kg/day or higher (i.e., 10^{0}–10^{2} mg/kg/day), as measured by the behavioral tests conducted. Three more recent studies of the cognitive effects of BPA were cited in the *ORNL* updated review. Two of these studies found no treatment effects on spatial memory or passive avoidance. In *Ryan et al (2006; also Ryan, 2005 dissertation)* no treatment effects were found for spatial memory (radial arm maze and Barnes maze) of ovariectomized juvenile female mice (males not tested) from dams dosed orally (gavage) with 0.002 or 0.2 mg BPA/kg/day from GD 3 to PND 21. Developmental exposure to the concomitant positive control, ethinyl estradiol, improved performance (decreased errors) in both spatial memory tasks. *Fujimoto et al (2006)* found no effects on a passive avoidance task in 8 week old male and female offspring of dams given BPA in drinking water (0.015 mg/kg/day) from GD 13 until parturition. In contrast to the study by *Ryan et al (2006), Xu et al (2007)* reported an increased response latency (deficit performance) in the spatial memory Morris water maze task for 10 week old male, but not female, offspring from dams given BPA in drinking water at a dose of 0.02 mg/kg/day, but not the

higher dose of 10 mg/kg/day, from GD 11 until PND 21. Due to questions about the experimental design of this study, including the adequacy of the vehicle control (refer to *ORNL* critique of this study), confirmation of this study's spatial memory findings would be needed before any conclusions could be drawn. One non-developmental study *(Maclusky et al., 2005)* in the *ORNL* review provides some additional information relevant to the issue of learning/memory effects of BPA. Ovariectomized adult female rats were dosed subcutaneously with combinations of estradiol and increasing doses of BPA (0, 0.04, 0.12, and 0.4 mg/kg). BPA dose-dependently inhibited the estradiol-induced formation of dendritic spine synapses on pyramidal neurons in the CA1 area of the hippocampus (an important brain region for memory/learning). However, the relevance of this information to possible effects of BPA on hippocampal development is unknown and to date, no comparable effects have been specifically reported in developing animals. Consequently, the additional behavioral studies cited in the ORNL updated review tend to support the determination that there is no consistent reliable evidence that developmental exposure to BPA causes adverse effects on offspring learning and memory at low or high doses, based on testing conducted to date.

Operant Behavior for Delayed Larger Reinforcers

Based on the limited available information on operant effects of BPA in the *Exponent* review of the literature, developmental exposure to BPA (0.04 mg/kg/day) via oral micropipette dosing appears to improve the ability of male and female rats to perform a complex operant task that is dependent on learning and memory. Pending confirmation, these results provide tentative supporting evidence that developmental exposure to BPA does not adversely affect cognitive behaviors in experimental offspring. The updated review conducted by *ORNL* revealed no additional relevant information on complex operant behaviors that would modify this conclusion.

Complex/Emotional Behaviors

Several authors evaluated complex behaviors that they related to "feelings" of impulsivity, stress and anxiety. There was a tendency to inappropriately anthropomorphize behaviors. However, for the purposes of this discussion, the conclusions of the authors are reported.

Stress/Anxiety

Several studies, reviewed by *Exponent*, used measures such as activity in a novel open environment and in the elevated plus maze test to assess "stress" and "anxiety" related behaviors, respectively, in offspring from dams exposed orally (micropipette dosing) to BPA (0.04 and 0.4 mg/kg/day). *Exponent* found no consistent effects to indicate that BPA affects stress or anxiety as measured by these tests. Three more recent studies were cited in the ORNL review that included an assessment of the effects of developmental exposure to BPA on offspring anxiety-like behaviors (*Fujimoto et al, 2006; Gioiosa et al, 2007; Ryan*

and Vandenbergh, 2006, also Ryan, 2005 dissertation). *Fujimoto et al (2006)* used the elevated plus maze to test adult male and female offspring of pregnant rats given BPA in drinking water at a dose of 0.015 mg/kg/day from GD 13 to parturition. *Gioiosa et al (2007)* also used the elevated plus maze for adult male and female offspring of pregnant mice dosed orally with 0.01 mg BPA/kg/day from GD 11 to PND 8. *Ryan and Vandenbergh (2006)* used two measures of anxiety-like behavior (the elevated plus maze and a light/dark preference chamber test) to test adult female offspring (males not tested) of pregnant mice dosed orally with 0.002 or 0.2 mg BPA/kg/day from GD 3 to PND 21. All three studies found no treatment related effects on elevated plus maze behaviors. In contrast to those negative findings, *Ryan and Vandenbergh* did report a significant effect in the light/dark preference test, specifically a dose-related decrease in time spent in the lighted chamber (significant for the 0.2 mg/kg/day dose of BPA); the positive control, ethinyl estradiol, used in this study also significantly decreased time in the lighted chamber. Decreased time in the lighted chamber was interpreted by the investigators as indicative of increased "anxiety-like" behavior. Overall, the additional studies cited in the *ORNL* updated review appear to support the determination that there are no consistent data to indicate that BPA affects stress or anxiety, as tested in these studies.

Pain-Related Behaviors/Opioid System

The underlying hypothesis regarding pain is that estrogens modulate the opioid system, which in turn, affects perception of pain. The few relevant studies available for *Exponent*'s review utilized the formalin paw test. Based on their review, *Exponent* suggested that further experiments are needed to reproduce the [limited] results with adequate litter size and to evaluate the validity of this animal model and its relevance to humans before any definitive conclusions can be made. Although the *ORNL* updated review found no additional studies measuring pain perception, two recent studies were reviewed *(Narita et al., 2006 and 2007)* that investigated possible BPA related modulation of the opioid system from a different perspective. Both of these studies, and one study reviewed by *Exponent (Mizuo et al., 2004)*, appear to be sequential reports of an interrelated project conducted within the laboratory of *Mizuo* and *Narita*. All three studies used dietary exposure of pregnant mice to BPA at various dose levels and conducted testing/analyses on adult male offspring only (females were not tested). *Mizuo et al (2004)* exposed pregnant mice to daily BPA doses of 0.4, 100 and 400 mg/kg/day from GD 0 until PND 23. A significantly increased hyperactivity response to morphine was found in adult offspring from dams treated with BPA at 400 mg/kg/day (the only BPA dose tested in this paradigm) and significant dose-related increases in conditioned place preference for morphine were found in offspring from dams treated with BPA at doses of 100 and 400 mg/kg/day (all three BPA doses were tested for place preference). Importantly, no changes were found in the μ-opioid mediated G-protein activation or μ-opioid receptor m-RNA in the limbic brain region. In the subsequent study by *Narito et al (2006)* mice were fed BPA in the diet at doses of 0.006, 0.06, 0.6, 100 and 400 mg/kg/day from GD 0 until PND 23 (data were shown only for the 0.006, 0.6 and 400 mg/kg/day dose groups). Comparable to the previous study, the adult offspring showed significant potentiation of morphine-induced hyperactivity and increased conditioned

place preference for morphine, both effects occurring at the low and high BPA dose levels of 0.006 and 400 mg/kg/day, but not at the middle dose of 0.6 mg/kg/day. Significant up-regulation of dopamine-mediated G-protein activation in the limbic forebrain was concomitantly found at all three dose levels. The third study in this series treated pregnant mice with a single BPA dose of 400 mg/kg/day but at four distinct phases of development, GD 0 to GD 7 (implantation), GD 7 to GD 14 (organogenesis), GD 14 to GD 20 (parturition) and PND 0 to PND 20 (lactation). As in the previous studies, the offspring showed potentiation of the morphine-induced hyperactivity and enhanced conditioned place preference for morphine but, significantly, only in offspring exposed to BPA during periods of organogenesis or lactation, not implantation or parturition. Confirmation of the findings from these latter studies by other laboratories using well-designed and controlled safety assessment protocols is needed before any definitive conclusions or extrapolations can be made about the potential effects of BPA on the functional development of the opioid system in conjunction with the limbic dopaminergic system.

Social Play/Non-social Behaviors

The study of general social play/non-social behaviors in animal models relies in large measure on the subjective monitoring of species specific behaviors. From a regulatory science perspective, without rigorous validation of these measures and a clear determination of the biological significance and human relevance of select increases and decreases in component social behaviors, findings based on these measures have very limited utility in the assessment of neurotoxic risk or in support of food additive regulatory decisions. *Exponent* pointed out that results for such behaviors from several studies have not been replicated in other laboratories or by the investigators themselves. These studies should not be interpreted as providing strong experimental data regarding the potential for BPA to cause adverse effects in behaviors that are directly relevant to human behaviors. The literature reviews conducted by *Exponent* and *ORNL* have revealed no consistent evidence of biologically significant adverse effects of developmental exposure to BPA on social play-related behaviors.

Aggression

Based on *Exponent's* literature review, the few available studies do not indicate that developmental BPA has a treatment-related effect on aggressive behavior. The updated review conducted by *ORNL* revealed no additional relevant information regarding the effects of BPA on aggressive behaviors that would modify this conclusion.

Socio-sexual Behavior

In the *Exponent* literature review, socio-sexual behaviors were considered outside the scope of the review on the effects of BPA on neurobehavioral endpoints. Nevertheless, several studies that reported measures of sexual behavior were included in that review. The range of effects on sexual behavior as noted in those studies was inconsistent, including no effects on male or female sexual behavior, reduced receptivity of females to males, and no effect on males but a slight

intensification of female sexual behavior. In the *ORNL* updated review, only one additional study *(Della Seta et al., 2006)* was cited in which the effects of BPA on male rat sexual behavior was specifically assessed (sexual behavior of the females was not assessed). This was a somewhat atypical developmental study in that male juvenile rats were orally dosed with 0.04 mg BPA/kg/day on PND 23 to PND 30 and subsequently tested for sexual behavior after PND 90. Ethinyl estradiol (EE) was included as a positive control. EE produced clear significant enhancement of male sexual behavior. BPA showed only a nominal increase in male sexual behavior, significant only for latency to intromission, one of eight measures of sexual behavior. With regard to the possible biological significance of such effects, *Exponent's* review pointed out that BPA has not been demonstrated to decrease reproductive function, as evidenced by the lack of findings at low dose levels in two large multi-generation reproduction studies *(Ema et al., 2001; Tyl et al., 2002). ORNL* concurs with *Exponent*. Overall, based on the limited number of studies and considering the limitation of these studies (refer to *Exponent's* and *ORNL* review comments), there appears to be no consistent or reliable evidence thus far that BPA adversely affects sexual behavior in the animal model used. In view of the clear effects of ethinyl estradiol *(Della Seta et al., 2006)* and in consideration of BPA's reported estrogenic effects, it would be well-served for more attention to be paid to assessing the utility of this particular endpoint (i.e. sexual behavior in male and female offspring) as a valuable index of potential developmental effects of BPA.

Maternal Behavior

In the *Exponent* literature review, only one study was found that evaluated maternal behaviors. Although the authors concluded that BPA significantly affected F1 maternal behaviors, the *Exponent* reviewer emphasized that none of the statistically significant changes in maternal behaviors were considered aberrant nor were there any adverse effects on the physical or functional development of the pups. Consequently, it was deemed that the experimental data from that study did not support the author's conclusion that BPA causes "adverse" effects on maternal behaviors. In the *ORNL* update review, two studies addressed maternal behavior but only one of those reported a specific assessment of the effects of developmental exposure on maternal behaviors in rats *(Della Seta et al., 2005)*. Pregnant dams were orally treated with 0.04 mg/kg/day from GD 1 to PND 21. Only one (duration of licking-grooming) of 14 indices of maternal behavior showed an overall significant effect of BPA treatment and several other indices showed marginal effects. The biological significance of this limited effect is questionable since there were no significant treatment effects on pup body weights. In the other study reviewed by *ORNL (Narita et al., 2006)* mice were fed diets with effective daily BPA doses of 0, 0.006, 0.06, 0.6, 100 and 400 mg/kg/day from GD 0 until PND 23. This study reported that there was no treatment related disruption of maternal behavior but did not present any data on how that determination was made other than the normal growth of the pups. Overall, based on the limited number of studies assessing the effects of BPA on

maternal behavior, there seems to be no clear evidence that developmental exposure to BPA causes adverse effects on maternal behavior.

Pharmacologic Challenge and Relevant Morphochemical Findings Related to Development of Dopaminergic/Monoaminergic Circuitry

There were 4 studies in the *Exponent* literature review that evaluated the effect of developmental BPA exposure on pharmacologically-induced behaviors. The primary underlying hypothesis being tested is that alteration of a pharmacologically-induced behavior could indicate that developmental exposures to BPA have effects on the developing nervous system, for example at the metabolic level, the level of neuronal circuitry organization, or the receptor level. These effects may have subtle consequences that are detectable in animal models only by evaluating changes in the functional response to a pharmacologic agent. While each of these studies did show that developmental exposure to BPA across a range of doses can modulate the behavioral response of adult offspring to pharmacologic challenge, there were conflicting results in the direction of that modulation. *Adriani et al. (2003)* reported that perinatal exposures to BPA (0.04 mg/kg/day) attenuated the amphetamine-induced hyperactivity in male, but not female, offspring. Amphetamine acts by increasing endogenous levels of dopamine and norepinephrine in the synapse. Consistent with the *Adriani* study, *Negishi et al. (2004)* also reported that BPA (0.1 mg/kg/day) attenuated the tranylcypromine-induced hyperactivity in male rats (females not tested). Tranylcypromine, monoamine oxidase inhibitor, also increases endogenous levels of dopamine and norepinephrine in the synapse. *Suzuki et al. (2003),* however, reported that developmental exposure to BPA in the diet at an estimated dose of 300 mg/kg/day (the only dose level of BPA tested in this paradigm) enhanced the methamphetamine-induced hyperactivity in male offspring (female offspring not tested). Pharmacologically, methamphetamine is similar to amphetamine and increases synaptic levels of dopamine and norepinephrine. However, *Suzuki et al. (2003)* additionally demonstrated that BPA can also potentiate the effects of methamphetamine in a different behavioral paradigm, the conditioned place preference test. They showed that developmental exposure to BPA in the diet at estimated dose levels of 0.03, 75 and 300 mg/kg/day enhanced the dopamine-dependent preference for methamphetamine in male offspring. Furthermore, these investigators also provided preliminary evidence that perinatal BPA exposure increased dopamine D1 receptor production in the brain. While the mechanisms underlying these apparently conflicting effects of BPA on pharmacologically induced behaviors is unclear, the fact that all three studies did show that BPA significantly modulated the response to pharmacological challenge in adult offspring does suggest that developmental exposure to BPA may have effects on monoaminergic neural pathways.

Similar experiments were conducted in *Suzuki's* laboratory to evaluate the effects of developmental exposure to BPA on morphine-induced effects on activity and conditioned place preference *(Mizuo et al. 2004)*, as described previously in the section on "*Pain-related Behaviors/Opioid System*". Dietary exposure to BPA from GD 0 to PND 23 potentiated a morphine-induced hyperactivity (300 mg BPA/kg/day; the only dose tested in this paradigm) and conditioned place preference (75 and 300 mg BPA/kg/day) in adult

male offspring (females not tested). The authors cited other literature suggesting dopamine's involvement in the pharmacologic responses to morphine. No additional pharmacologic challenge studies were cited in the *ORNL* updated review but several recent immunohistochemical and biochemical studies were cited that provided some correlative information about the effects of developmental BPA exposure on tyrosine hydroxylase (a dopamine/monoamine synthesizing enzyme), dopamine activation of G-related proteins, neurotransmitter levels and the expression of brain dopaminergic receptor mRNA.

Three investigators reported increased dopaminergic/monoaminergic related activity. *Narito et al. (2006)* fed mice diets with effective daily BPA doses of 0, 0.006, 0.06, 0.6, 100 and 400 mg/kg/day from GD 0 until PND 23 (data were shown only for the 0.006, 0.6 and 400 mg/kg/day dose groups). Significant up regulation of dopamine mediated G-protein activation in the limbic forebrain was found at the 0.006, 0.6 and 400 mg/kg/day dose levels of BPA (the same dose levels that potentiated the hyperactivity and conditioned place preference for morphine as reported in this study). *Patisaul et al. (2006, 2007)* treated male neonates subcutaneously *(note not orally)* with 0.2 mg BPA/day (calculated dose of 50 mg/kg) from PND 1 to PND 2 and found increased tyrosine hydroxylase in the anteroventral periventricular (AVPV) nucleus of the preoptic area in PND 19 pups. At PND 85, the gonadectomized males demonstrated an enhanced progesterone/estrogen-induced increase in calbindin in the sexually-dimorphic nucleus of the preoptic area. *Honma et al. (2006)* treated pregnant rats orally with 0, 4, 40 and 400 mg BPA/kg/day from GD 6 to PND 20 and measured regional brain levels of monoaminergic neurotransmitters. The authors reported that the turnover of dopamine and serotonin was increased in female offspring at the 4 and 40 mg/kg/day dose levels in varying brain regions (pregnant rats dosed with 400 mg/kg/day died and no offspring analyses were conducted). Several other investigators, however, reported findings suggesting decreased dopaminergic/monoaminergic activity. *Ishido et al. (2007)* treated rat male neonates orally with 0.6 mg BPA/pup (calculated dose of 12-60 mg/kg/day) from PND 5 to PND 21. A significant decrease in tyrosine hydroxylase (TH) was found in the substantia nigra with a corresponding increase in apoptotic activity (i.e., cell degeneration) and a complete inhibition of dopamine transporter gene expression. *Rubin et al. (2006)* also reported decreased tyrosine hydroxylase neurons in the AVPV of the preoptic area of female but not male adult offspring from mice exposed subcutaneously via an indwelling osmotic pump *(note not orally)* with 0.250 mcg BPA/kg/day from GD 8 to PND 16. *Kawai et al. (2007)*, dosing pregnant mice orally with 0.002 mg/kg/day from GD 11 to GD 17, reported no effect on male offspring serotonin or serotonin transporter in the dorsal raphe nucleus (females not tested). Finally, *Tando et al. (2007)* fed BPA to pregnant mice in the diet at effective doses of 0, 0.6 and 1600 mg/kg/day from GD 0 to PND 21. The authors report significant decreases in tyrosine hydroxylase of the substantia nigra for adult female offspring (not males) at the lower 0.6 mg/kg/day dose of BPA but no significant effects at the higher dose.

However, as detailed in the *Exponent* and *ORNL* reviews, various limitations in experimental design and uncertainties regarding the data were noted for each of these studies. Consequently, it is premature to make conclusions about the significance of these pharmacologic challenge and cytochemical studies or their human relevance without

confirmation of these reported findings in different laboratories using well-designed safety assessment protocols. Viewing the findings of these studies collectively, however, they appear to provide tentative pharmacologic, behavioral and cytochemical results that suggest one potential mode of action for developmental exposure to BPA is modulation of the dopaminergic and/or other monoaminergic receptor systems.

Hormone Levels and Related Biochemical Factors

There was no study information described in the *Exponent* review that dealt with hormone levels or other biochemical measures. Several studies in the *ORNL* updated review did include such measures. Three studies presented data on testosterone showing decreased levels in male offspring, two studies finding statistically significant changes. *Della Seta et al. (2006)* treated male rats orally with 0.04 mg BPA/kg/day from PND 23 to PND 30. Significant decreases in testosterone levels were found in juvenile (PND 37) and adult (PND 105) male offspring (females not tested). *Ceccarelli et al. (2007)* treated male and female juvenile rats orally with 0.04 mg BPA/kg/day from PND 23 to PND 30. Significantly decreased testosterone levels were reported in juvenile males (PND 37) but not adult (PND 90) males. BPA had no effects in females or on estradiol levels in either sex. Interestingly, the positive control, ethinyl estradiol, significantly increased testosterone levels in PND 37 female offspring. *Kawai et al. (2007)* treated pregnant mice orally with 0.002 mg BPA/kg/day from GD11-17 but found only a nonsignificant trend for decreased testosterone levels in adult male offspring (females not tested). Several other studies considered the possible developmental effects of BPA on thyroid function and related treatment effects. *Xu et al. (2007)* exposed pregnant rats to drinking water with calculated doses of 0.02 and 10 mg BPA/kg/day from GD 11 to PND 21. Significantly elevated thyroxin (T4) levels were found for the low dose (0.02 mg/kg/day) male offspring at PND 7, but then at PND 21, the T4 levels decreased at the high dose (10 mg/kg/day). Female offspring were not affected. Dams in the 0.02 mg/kg/day dose group had decreased T4 levels on PND 7. Expression in brain of RC3/neurogranin (a thyroid hormone responsive gene) and thyroid hormone receptors α and β (THR-α/β) mRNA was unchanged but expression of steroid hormone receptor coactivator 1 (SHC-1) mRNA was significantly elevated in the 0.02 mg/kg/day (higher dose not tested) male pups at PND 5 and PND 7 (females not tested). *Zoeller et al. (2005)* fed pregnant dams food wafers with BPA at dose levels of 1, 10 or 50 mg/kg/day from GD 6 to PND 21. Significantly elevated T4 levels were found for both male and female pups in all three dose groups on PND 15 (but not PND 4, 8 or 35). A correlated effect, also in males at all dose levels, was significantly increased expression of RC3/neurogranin mRNA specifically on PND 15, as well. Thyroid stimulating hormone levels were not affected. Since a positive estrogenic control was not used, it is not known whether the elevated T4 was associated with the estrogenic effects of BPA or some other mechanism.

Several morphochemical and gene expression studies were included in the *ORNL* review that may possibly relate to the potential influence of BPA on thyroid function, *Nakamura et al. (2007)* treated mice subcutaneously (note not orally) with 0.02 mg BPA/kg/day over different periods of gestation, specifically GD 0 to 12, GD 0 to 14, and GD 0 to 16, and found that the pattern of neuronal differentiation/migration was altered in neocortical,

as well as thalamocortical connections, which the authors suggest are similar to those associated with thyroid dysfunction. The functional significance of these particular changes is unknown. *Nishizawa et al. (2005a and 2005b)* studied aryl hydrocarbon (AhR) and retinoid receptors (RARα and RXRα), xenobiotic metabolizing enzymes (XMEs) and related factors in rat embryos from dams exposed to BPA orally at doses of 0.00002, 0.002, 0.2 and 20 mg/kg/day spanning the period of GD 6 to GD 17. BPA dose-independently (variable U-function dose response) upregulated expression of virtually all of these receptor mRNAs (and increased liver protein levels for several factors) during embryogenesis. The authors suggested that the BPA related changes in RARα and RXRα may disrupt thyroid hormone receptor-mediated transcription.

Two other studies in the *ORNL* review touched on the potential effects of developmental BPA exposure on other neuronal systems. In juvenile and adult female offspring (males not tested) of pregnant rats dosed orally with 0.4 mg BPA/kg/day through gestation and lactation, *Facciolo et al. (2005)* found highly significant treatment effects on mRNA expression of the neurotransmitter receptors, somatostatin and α $GABA_A$, but the directions of these effects (i.e., increased or decreased) were highly variable across various parts of the brain. *Funabashi et al. (2004)* exposed pregnant rats to 2.5 mg BPA/kg/day via drinking water through gestation and lactation. No treatment related changes in number of corticotrophin-releasing hormone (CRH) neurons were found in either the preoptic area (POA) or the bed nucleus of the stria terminalis (BST) for either adult male or female offspring. However, in control animals, the number of CRH neurons in both POA and BST for females significantly exceeded that for males. In the BPA exposed offspring, this sex difference was no longer observed in the BST. The physiological significance of this is not known.

While several of these studies did suggest interesting findings for developmental exposure to BPA related to offspring testosterone levels and thyroid function, and one study suggested an influence of BPA on somatostatin and GABA brain receptors, the *ORNL* critique describes various limitations in experimental design and uncertainties regarding the data for each of these studies. Consequently, it is premature to make conclusions about the significance of these findings or their human relevance without confirmation in different laboratories using well-designed safety assessment protocols.

Factors Related to Sexual Development and Differentiation

Sexually Dimorphic Brain Morphology and Cytochemistry

In the *Exponent* literature review, two papers from a single laboratory *(Kubo et al., 2001* and *2003*) focusing on morphology of discrete sexually dimorphic regions of the brain reported that gestational and lactational exposure of rat dams to BPA in drinking water at estimated dose levels of 0.03 and 0.3 mg/kg/day (*Kubo et al., 2003*) and 1.5 mg/kg/day (*Kubo et al., 2001*) altered the sexual differences in volume and number of neurons of the locus coeruleus between male and female offspring (F>M in controls; F=M or F<M in BPA groups), but did not affect similar male/female differences in the sexually dimorphic nucleus of the

preoptic area (SDN-POA). The positive control, diethylstilbestrol had a similar effect. The experimental treatment tended to increase the size of the locus coeruleus in males and decrease the size in females. However, it is unclear whether such treatment related effects in the males and females were actually statistically significant. Due to several experimental design limitations (refer to *Exponent* review and critique report) and the fact that these studies are from a single laboratory, these results should be considered preliminary findings that need to be replicated in other laboratories using well-designed safety assessment protocols.

Several papers in the *ORNL* updated review used various cytohistochemical methods, including fluorescent markers and analysis of the expression of mRNAs for select receptors, to assess morphochemical effects of BPA on regional brain areas. In the study by *Nakamura et al. (2007)*, fetuses of pregnant mice dosed subcutaneously (note not orally) with 0.02 mg BPA/kg/day during gestation had altered patterns of neuronal differentiation/migration in the neocortical, as well as thalamocortical, connections of the brain. *Ceccarelli et al. (2007)* dosed juvenile male and female rats orally with 0.04 mg BPA/kg/day from PND 23 to PND 30. BPA treatment significantly affected the numbers of neurons with estrogen receptor α (ER α) in various regions of the hypothalamus. On PND 37, female offspring showed increased ER α neurons in the ventromedial nucleus and the medial preoptic nucleus; PND 37 males showed increased ER α cells in the arcuate area. No effects were noted in any hypothalamic area in BPA treated animals at PND 90. The positive estrogenic control, ethinyl estradiol, mimicked these effects of BPA with some additional effects. Interestingly, control animals showed significant sex differences in numbers of ER α cells in the arcuate nucleus (M>F) at both PND 37 and 90, the ventromedial nucleus (F>M) at PND 90 only, and the medial preoptic area (M>F) at PND 37. The statistically significant BPA induced increase in female ER α cells in the medial preoptic area prevented the expression of sexual dimorphism in that area. The functional significance of these M/F differences in ER α distributions is not known. *Kawai et al. (2007)* also reported increased ER α, as well as ER β, cells in the dorsal raphe nucleus of adult male offspring from pregnant mice given BPA orally at a dose of 0.002 mg/kg/day from GD 11 to GD 17. This study also reported that there were no corresponding changes in the levels of serotonin or the serotonin transporter in the dorsal raphe nucleus, and no changes in serum testosterone.

Several other studies reported BPA related changes in tyrosine hydroxylase immunoreactivity (TH-ir) in sexually dimorphic brain regions in which TH-ir is greater in females than males. *Patisaul et al. (2006, 2007)* dosed male neonates subcutaneously *(note not orally)* with 0.5 mg BPA/day (calculated dose of 50 mg/kg) from PND 1 to PND 2 and found increased tyrosine hydroxylase in the anteroventral periventricular nucleus of the preoptic area in PND 19 pups. Also, at PND 85, gonadectomized adult BPA males demonstrated an enhanced progesterone/estrogen-induced increase in calbindin in the sexually dimorphic nucleus of the preoptic area. *Rubin et al. (2006)* also reported decreased tyrosine

hydroxylase neurons in the AVPV of the preoptic area of female but not male adult mice from dams dosed subcutaneously via an indwelling osmotic pump *(note not orally)* with 0.25 mcg BPA/kg/day from GD 8 to PND 16. *Ishido et al. (2007)* dosed rat male neonates orally with 0.6 mg BPA/pup (calculated dose of 12-60 mg/kg/day) from PND 5 to PND 21. A significant decrease in tyrosine hydroxylase (TH) was found in the substantia nigra with a corresponding increase in apoptotic activity (i.e., cell degeneration) and a complete inhibition of the dopamine transporter gene expression. Finally, *Tando et al. (2007)* fed BPA to pregnant mice in the diet at effective doses of 0, 0.6 and 1600 mg/kg/day from GD 0 to PND 21. The authors report significant decreases in tyrosine hydroxylase of the substantia nigra for adult female offspring (not males) in the lower 0.6 mg/kg/day dose group of BPA but no significant effects at the higher dose.

While several of these studies suggest interesting findings for early exposure to BPA related to the development of sexually dimorphic features of brain morphology and cytochemical profiles, the *Exponent* and *ORNL* reviews describe various limitations in experimental design and uncertainties regarding the data for each of these studies. Consequently, it is premature to make conclusions about the significance of these findings or their human relevance without confirmation of these reported findings using well-designed safety assessment protocols.

Sexually Dimorphic Behavioral Measures

Typically, the basis for determining that there is a sexual difference in behavior is that a behavioral measure (e.g., activity) in control females is statistically different (higher/lower) from that of control males. In animals exposed to treatment (e.g., BPA), the absence of statistically significant difference between treated males and females is taken as evidence of decreased sexual differentiation, even though there may be no significant treatment effects in males or in females when compared with their respective controls. In the *Exponent* literature review, several papers were cited that presented findings related to sexually dimorphic behavior. These papers appeared to provide little consistent or replicated findings showing that BPA reduces sexual differences in behavior, specifically activity, learning/memory, and social, non-social and sexual behaviors. The *ORNL* updated review also cited three studies that assessed sex related differences in behavior and all three of these studies reported BPA-related impaired sex differentiation. *Gioiosa et al. (2007),* testing juvenile and adult offspring from pregnant mice treated orally with 0.01 mg BPA/kg/day from GD 11 to PND 8, found control animals to exhibit sexually dimorphic behaviors for various measures in tests of novelty seeking, open field exploration, and the elevated plus maze. In the BPA offspring these sex related differences were diminished or eliminated with female behaviors resembling those of males. *Fujimoto et al. (2006)* gave drinking water to pregnant rats with a dose of 0.015 mg BPA/kg/day from GD 13 to parturition and tested offspring in a series of behavioral tests including open field, elevated plus maze, passive avoidance and a forced swim task. Controls exhibited significant sex related differences in open field rearing and forced swim task struggling behavior (F>M in both tests). In BPA exposed offspring, these sex

differences were eliminated such that F=M, due primarily to significant increases in male rearing and immobility scores. No sex differences, or BPA effects, were noted for the elevated plus maze or the passive avoidance test. Finally, ***Rubin et al. (2006)*** also reported a BPA-related alteration of sexually dimorphic behaviors in the open field (female behavior becoming more like that of male) of adult mice from dams dosed subcutaneously via an indwelling osmotic pump ***(note not orally)*** with 0.25 mg BPA/kg/day from GD 8 to PND 16. Notably, this functional measure of BPA altered sexual differentiation was accompanied by a decrease in the sexually dimorphic number of tyrosine hydroxylase neurons in the AVPV of the preoptic area of females, but not males. In addition to ***Rubin et al. (2006)*** several other studies in the *ORNL* updated review (refer also to sections above, "*Sexually Dimorphic Brain Morphology and Cytochemistry*" and "*Pharmacologic Challenge and Relevant Morphochemical Findings Related to Development of Dopaminergic/Monoaminergic Circuitry*") did provide relatively recent data consistent with the suggestion that BPA could affect sexual differentiation. These studies showed that developmental exposure to BPA may modulate the development of sexually dimorphic areas of the brain, for example, in terms of changes in number/distribution of cells expressing estrogen receptors or tyrosine hydroxylase immunoreactivity. However, the specific functional (behavioral) significance of those purported morphochemical changes has not yet been established.

While the above studies do present a number of positive and interesting findings for possible effects of BPA on brain development and sexually dimorphic behaviors, both the *Exponent* and *ORNL* reviews/critiques describe various limitations in experimental design and uncertainties regarding the data for each of these studies. Consequently, it is premature to make conclusions about the significance of these findings or extrapolate their human relevance without confirmation of these effects using well-designed safety assessment protocols and clarification of the biological significance of the BPA related morphochemical changes in sexually dimorphic regions of the brain and changes in sexually dimorphic behavioral functions.

Executive Summary and Overall Conclusions

In the first task of this assignment, the report titled "*Exponent: Literature Review of Neurobehavioral Effects of Bisphenol A*" was reviewed for accuracy and confidence in the conclusions. Overall, the reviewers agree with the summaries and conclusions presented throughout the document. The second task in this assignment was the conduct by *ORNL* of an updated review of the scientific literature on the neurotoxicity of bisphenol A (BPA), published since the document in Task 1 was completed. The search strategy of the ORNL review was designed to limit the findings to studies which assessed neurotoxicity or developmental neurotoxicity endpoints. Studies were found in which testing was conducted on the treated animals and in which testing was conducted on the offspring following maternal treatment. Endpoints included both behavioral and morphochemical measurements. Preference was given to studies which used oral administration but relevant information from other studies was considered.

Exponent and *ORNL* reviewed a total of 49 papers, published between 2001 through 2007, which investigated BPA's potential neurobehavioral toxicity following developmental exposure. Most studies included exposure levels below 50 mg/kg/day (*EPA's* LOAEL). The oral route of exposure (gavage, micropipette, diet, drinking water) was used in most studies (37), with the remainder using parenteral dosing (subcutaneous, intracisternal, intracerebral). A variety of exposure regimens were used in which animals were given BPA at different time periods during gestation, lactation and/or after weaning. Approximately 26 of the oral studies exposed dams to BPA throughout the entire gestation and lactation developmental periods; the remaining studies used exposures of 1 day to several weeks during select periods of development.

It should be clear that, in the opinion of the reviewers, virtually all of the studies in the *ORNL* updated review (31), and most of those in the *Exponent* review (18), have a variety of basic experimental design shortcomings which impact to various degrees the confidence in the data and/or confound the interpretability of the study findings from individual studies. These shortcomings include such factors as no available dose response information (24/49 studies used only a single dose of BPA), low numbers of experimental subjects per group, selective use of only male or female offspring for testing, inadequate control procedures, lack of positive controls, absence of correlative morphochemical and functional endpoints, and failure to consider litter as the appropriate statistical unit (refer to the *Exponent* and *ORNL* critiques of the studies included in their reviews). Also, the procedure of limiting exposure to only select portions (days or weeks) of the developmental period, for example during critical periods of nervous system development, which was used in a number of the studies reviewed, may be of use in mechanistic studies of developmental neurotoxicity or, possibly, in the safety assessment of certain types of substances with expected human exposures occurring only during discrete periods of development, but the use of limited periods of exposure does not provide an accurate assessment of a test substance's potential developmental neurotoxicity throughout the period of development which is typically needed for food-related safety assessments. In addition, since select critical periods may occur at various times during development, the variety of exposure regimens used may have contributed

to some of the inconsistent or conflicting findings reported in the reviewed studies on developmental neurotoxic potential of BPA.

Consequently, in view of the limitations in study design resulting in questionable confidence in the data and/or confounding the interpretability of the study findings, without appropriate confirmation of these findings using well-designed experimental protocols and clarification of their biological significance, none of these studies may be used individually for supporting safety assessment determinations or regulatory decisions. However, with the above caveats in mind the varied treatment related findings in a majority of the reviewed studies do collectively present a weight of evidence generally supporting the contention that BPA has a potential for selectively altering the morphochemical development of the brain, including sexually dimorphic regions, and certain behavioral responses of juvenile and adult offspring. However, until these disparate findings can be replicated in well-designed safety assessment studies using sensitive biomarkers of effect, it is not possible to specify reliably the nature and extent of the BPA related effects, their biological significance, or the LOAEL and NOAEL of BPA.

Among the findings reported in these studies, a number of behaviors were identified for which the reviewers found little or no clear or consistent credible evidence of significant effects of BPA treatment in juvenile or adult experimental offspring. These included ontogeny of sensory/motor behaviors and reflexes, self-grooming, open-field defecation scores, social play/non-social behaviors, aggression, stress/anxiety, and maternal behavior. Behavioral measures of learning and memory were also found to show no consistent reliable evidence of adverse effects in experimental offspring, although schedule-controlled operant behavior was reported as being improved in rat offspring. There was also no consistent evidence that BPA adversely affects sexual behavior in rodents. However, in view of the clear effects of ethinyl estradiol (a positive estrogenic control) in enhancing male sexual behavior and in consideration of BPA's reported estrogenic effects, it would be well-served for more attention to be paid to assessing the utility of this endpoint (i.e., sexual behavior) as an index of potential developmental effects of BPA. There were also equivocal findings of BPA related changes in sexually dimorphic behaviors.

A number of studies did report findings of interest that collectively appear to suggest several general types of effects that might be attributable to developmental exposure to BPA: (1) the possible effects of BPA on morphochemical development of brain and sexual differentiation are suggested by preliminary findings of altered patterns of neuronal differentiation and migration in neocortical and thalamocortical connections, sex-dependent changes in the number of neurons in the locus coeruleus, and altered distribution of neurons with estrogen receptors or tyrosine hydroxylase immunoreactivity in sexually-dimorphic regions of the brain in offspring of BPA treated dams; (2) altered endocrine function in offspring of BPA exposed dams is suggested by preliminary reports of decreased testosterone levels in male offspring, altered thyroxin levels in postnatal pups, and conflicting reports of changes in expression of RC3/neurogranin mRNA (a thyroxine responsive gene) and steroid hormone receptor coactivator-1 mRNA; and (3)

the possibility that developmental exposure to BPA may modulate the development of the monoaminergic neural pathways is suggested by preliminary findings of significant changes in the behavioral responses of adult offspring to challenge with dopaminergic/noradrenergic pharmacologic agents (amphetamine, tranylcypromine and methamphetamine) and a series of immunohistochemical and biochemical studies of the effects of BPA on developmental distribution of tyrosine hydroxylase neurons, dopamine activation of G-related proteins, neurotransmitter levels, and the expression of brain dopamine receptor and dopamine transporter mRNA. These various findings of interest could help identify sensitive biomarkers for more definitive assessment of BPA's potential developmental neurotoxicity. However, in view of the caveats regarding limitations in experimental design and the questionable confidence in the data and their interpretability (see *Exponent* and *ORNL* review comments), it is premature to make firm conclusions about the utility and significance of these findings without appropriate confirmation of the findings using well-design experimental protocols and clarification of their biological relevance.

For purposes of completion, among the studies reviewed, the lowest observed effect level for offspring from dams treated with BPA is 0.002 mg/kg/day based on decreased sensitivity of rats to estradiol as measured by motor activity (*Ryan 2005; Ryan et al., 2006*) and increased estrogen receptor expression in male mice (*Kawai et al., 2007*); a no-observed adverse effect level was not identified; although lower doses were used in other studies, the endpoints were mechanistic in nature and of unknown relevance. A general comment should be made about the designation of LOAELs. Although each of the referenced studies included in this updated review reported some treatment related findings, it is questionable whether the designation of LOAEL should be applied in any of those studies that used only one dose/treatment level or were not adequately designed as food additive safety assessment studies. For those studies using only one dose/treatment level it is suggested that an alternate designation, such as Single Dose Study Effect Level (SDSEL), be considered to clearly designate that the effect level was from a single dose study. With regard to those research studies not specifically designed for safety assessment, a LOAEL (as well as NOAEL) designation should be applied only when the study findings are confirmed using a well-designed food additive safety assessment protocol that will also provide sufficient dose-response information to reliably estimate a LOAEL and NOAEL for BPA.

RECOMMENDATIONS FOR SUBSEQUENT STUDY

It should be noted that, while experimental design is important for any study conducted for safety assessment, the safety assessment of endocrine disruptors (e.g., bisphenol A and other estrogen disruptors) necessitates that particular attention be given to certain aspects of study design. For example, species and strain differences in the sensitivity to estrogens makes the selection of test subject and the use of appropriate positive controls to gauge the sensitivity of the test model very important design elements. The use of a sufficient range of dose levels is particularly important for endocrine disruptors to define as accurately as possible the dose-response relationships, including nonmonotonic, and to

identify the lowest observed adverse effect level (LOAEL) and the no observed adverse effect level (NOAEL). Dosing should also be included that most closely approximates expected human exposure. For the safety assessment of food related chemicals, the oral route of exposure is typically deemed the most appropriate and the period of chemical treatment should include at least the gestational and lactational periods of nervous system development. Care must be taken to avoid confounding effects of potentially direct exposure of offspring from food or drinking water during lactation. For potential endocrine disruptor chemicals, additional consideration should be given to extending the period of exposure to include the juvenile period of nervous system development, as well. Since estrogenic types of chemicals may occur as contaminants in a variety of materials (including animal foods, water and polycarbonate containers), it is important to minimize inadvertent exposure of experimental animals to such sources of chemical contaminants by using certified food, filtered water, appropriate bedding material, and non-polycarbonate caging or water bottles. The assessment of neurodevelopmental effects of early exposure to suspect endocrine disruptors should consider measures of morphochemical development of the brain, including the sexually dimorphic brain regions, sensitive behavioral measures, sexual behaviors, hormone level analyses, and the use of specific pharmacologic challenges to reveal subtle neurobehavioral dysfunction. To enhance the appropriate interpretation of such data, it is very important that all endpoints and the methods for their assessment be clearly defined, valid and relevant, that both sexes of offspring be fully evaluated, and that correlative morphochemical, endocrine, and neurochemical measures be included in the study to the extent possible. The preliminary findings of interest from the *Exponent/ORNL* literature report could be reviewed for possible sensitive biomarkers.

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Funabashi, T., Kawaguchi, M., Furuta, M., Fukushima, A., and Kimura, F. (2004) Exposure to bisphenol A during gestation and lactation causes loss of sex difference in corticotrophin-releasing hormone-immunoreactive neurons in the bed nucleus of the stria terminalis of rats. PNEC 29:475-485.

Gioiosa, L., Fissore, E., Ghirardelle, G., Parmigiani, S., and Palanza, P. (2007) Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. Horm. Behav. 52:307-316.

Honma, T., Miyagawa, M., Suda, M., Wang, R.-S., Kobayashi, K., and Sekiguchi, S. (2006) Effects of perinatal exposure to bisphenol A on brain neurotransmitters in female rat offspring. Indus. Health 44:510-524.

Ishido, M., Morita, M., Oka, S., Masuo, Y. (2005) Alteration of gene expression of G protein-coupled receptors in endocrine disruptors-caused hyperactive rats. Reg. Peptides 126:145-153.

Ishido, M., Yomemoto, J., Morita, M. (2007) Mesencephalic neurodegeneration in the orally administered bisphenol A-caused hyperactive rats. Toxicol. Lett. 173:66-72.

Kawai, K., Murakami, S., Senba, E., Yamanaka, T., Fujiwara, Y., Arimura, C., Nozaki, T., Takii, M., and Kubo, C. (2007) Changes in estrogen receptors α and β expression in the brain of mice exposed prenatally to bisphenol A. Reg. Toxicol. Pharmacol. 47:166-170.

Laviola, G., Gioiosa, L., Adriani, W., Palanza, P. (2005) D-Amphetamine-related reinforcing effects are reduced in mice exposed prenatally to estrogenic endocrine disruptors. Brain Res. Bull. 65:235-240.

MacLusky, N., Hajszan, T., Leranth, C. (2005) The environmental estrogen bisphenol A inhibits estradiol-induced hippocampal synaptogenesis. Environ. Health Persp. 113:675-679.

Masuo,Y., Morita, M., Oka, S., and Ishido, M. (2004a) Motor hyperactivity caused by a deficit in dopaminergic neurons and the effects of endocrine disruptors: a study inspired by the physiological roles of PACAP in brain. Reg. Peptides 123:225-234.

Masuo, Y., Ishido, M., Morita, M., and Oka, S. (2004b) Effects of neonatal treatment with 6-hydroxydopamine and endocrine disruptors on motor activity and gene expression in rats. Neural Plasticity 11:59-76.

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Mizuo, K., Narita, M., Miyagawa, K., Narita, M., Okuno, E., and Suzuki, T. (2004) Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice. Neurosci. Lett. 356:95-98.

Nakamura, K., Itoh, K., Yaoi, T., Fujiwara, Y., Sugimoto, T., and Fushiki, S. (2006) Murine neocortical histogenesis is perturbed by prenatal exposure to low doses of bisphenol A. J. Neurosci. Res. 84:1194-1205.

Nakamura, K., Itoh, K., Sugimoto, T., and Fushiki, S. (2007) Prenatal exposure to bisphenol A affects adult murine neocortical structure. Neurosci. Lett. 420:100-105.

Narita, M., Miyagawa, K., Mizuo, K., Yoshida, T., and Suzuki, T. (2006) Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect. Neurosci. Lett. 402:249-252.

Narita, M., Miyagawa, K., Mizuo, K., Yoshida, T., and Suzuki, T. (2007) Changes in central dopaminergic systems and morphine reward by prenatal and neonatal exposure to bisphenol-A in mice: evidence for the importance of exposure period. Addict. Biol. 12:167-172.

Negishi, T., Kawasake, K., Suzaki, S., Maeda, H., Ishii, Y., Kyuwa, S., Kuroda, Y., and Yoshikawa, Y. (2004) Behavioral alteration in response to fear-provoking stimuli and tranylcypromine induced by perinatal exposure to bisphenol A and nonylphenol in male rats. Environ. Health Perspect. 112:1159-1164.

Nishizawa, H., Morita, M., Sugimoto, M., Imanishi, S., and Manabe, N. (2005a) Effects of in utero exposure to bisphenol A on mRNA expression of arylhydrocarbon and retinoid receptors in murine embryos. J. Reprod. Dev. 51:315-324.

Nishizawa, H., Imanishi, S., and Manabe, N. (2005b) Effects of exposure in utero to bisphenol A on the expression of aryl hydrocarbon receptor, related factors, and xenobiotic metabolizing enzymes in murine embryos. J. Reprod. Dev. 51:593-605.

Patisaul, H.B., Fortino, A.E., and Polston, E.K. (2006) Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV. Neurotoxicol. Teratol. 28:111-118.

Patisaul, H.B., Fortino, A.E., and Polston, E.K. (2007) Differential disruption of nuclear volume and neuronal phenotype in the preoptic area by neonatal exposure to genistein and bisphenol-A. Neurotoxicol. 28:1-12.

Porrini, S., Belloni, V., Della Seta, D., Farabollini, F., Giannelli, G., and Dessì-Fulgheri, F. (2005) Early exposure to a low dose of bisphenol A affects socio-sexual behavior of juvenile female rats. Brain Res. Bull. 65:261-266.

Razzoli, M., Valsecchi, P., and Palanza P. (2005) Chronic exposure to low doses bisphenol A interferes with pair-bonding and exploration in female Mongolian gerbils. Brain Res. Bull. 65:249-254.

Rubin, B.S., Lenkowski, J.R., Schaeberle, C.M., Vandenberg, L.N., Ronsheim, P.M., and Soto, A.M. (2006) Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of bisphenol A. Endocrinol. 147:3681-3691.

Ryan, B.C. (2005) Developmental exposure to environmental estrogens alters adult behavior in female rodents. Doctoral thesis, North Carolina State University.

Ryan, B.C., Howdeshell, K.L., and Gray, L.E., Jr. (2006) Sexually dimorphic lordosis behavior is more sensitive to disruption by developmental exposure to ethinyl estradiol than is reproductive morphology in the Long Evans female rat. Biol. Reprod. (special issue) 134-135.

Ryan, B.C. and Vandenbergh, J.G. (2006) Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. Horm. Behav. 50:85-93.

Shikimi, H., Sakamoto, H., Mezaki, Y., Ukena, K., and Tsutsui, K. (2004) Dendritic growth in response to environmental estrogens in the developing Purkinje cell in rats. Neurosci. Lett. 364:114-118.

Tando, S., Itoh, K., Yaoi, T., Ikeda, J., Fujiwara, Y., and Fushiki, S. (2007) Effects of pre-and neonatal exposure to bisphenol A on murine brain development. Brain Develop. 29:352-356.

Xu, X., Liu, Y., Sadamatsu, M., Tsutsumi, S., Akaike, M., Ushijima, H., and Kato, N. (2007) Perinatal bisphenol A affects the behavior and SRC-1 expression of male pups but

does not influence on the thyroid hormone receptors and its responsive gene. Neurosci. Res. 58:149-155.

Zoeller, R.T., Bansal, R., Parris, C. (2005) Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist *in vitro*, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinol. 146:607-612.

APPENDIX B: Critique of Papers Used in ORNL Updated Review of Developmental Neurotoxicity Potential of Bisphenol A (BPA)

Thomas J. Sobotka, Ph.D.
March 31, 2008

As part of the updated review, each study will be critiqued for scientific merit (positive and negative aspects of the study impacting interpretability), relevance of findings to humans and a summary statement of the utility of the study results for supporting a food additive regulatory decision. It should be noted that, while experimental design is important for any study conducted for safety assessment, the safety assessment of endocrine disruptors (e.g., bisphenol A and other estrogen disruptors) necessitates that particular attention be given to certain aspects of study design. For example, species and strain differences in the sensitivity to estrogens makes the selection of test subject and the use of appropriate positive controls to gauge the sensitivity of the test model very important design elements. The use of a sufficient range of dose levels is particularly important for endocrine disruptors to define as accurately as possible dose-response relationships, including nonmonotonic, and to identify the lowest observed adverse effect level (LOAEL) and the no observed adverse effect level (NOAEL). Doses that most closely approximate expected human exposure should be included. For the safety assessment of food related chemicals, the oral route of exposure is typically deemed the most appropriate. Since estrogenic types of chemicals may occur as contaminants in a variety of materials (including animal foods, water and polycarbonate containers), it is important to minimize inadvertent exposure of experimental animals to such sources of chemical contaminants by using certified food, filtered water, appropriate bedding material, and non-polycarbonate caging or water bottles. The assessment of neurodevelopmental effects of early exposure to suspect endocrine disruptors will increasingly include measures of sexually dimorphic brain nuclei and behaviors and the use of specific pharmacologic challenges to reveal subtle neurobehavioral dysfunction. To enhance the appropriate interpretation of such data, it is very important that all endpoints and the methods for their assessment be clearly defined, valid and relevant, and that correlative morphochemical, endocrine, and neurochemical measures be included in the study to the extent possible.

Finally, there is one general comment that should be made about the designation of LOAELs. Although each of the referenced studies included in this updated review reported some treatment related findings, it is questionable whether the designation of LOAEL should be applied in any of those studies that used only one dose/treatment level or were not adequately designed as food additive safety assessment studies. For those studies using only one dose/treatment level, it is suggested that an alternate designation, such as Single Dose Study Effect Level (SDSEL), be considered to clearly designate that the effect level was from a single dose study. With regard to those studies not specifically designed for safety assessment a LOAEL (as well as NOAEL) designation should be applied only when the study findings are confirmed using a well-designed food additive safety assessment protocol that will also provide sufficient dose-response information to reliably estimate a LOAEL and NOAEL for BPA.

The following papers, used in the ORNL Updated Review of Developmental Neurotoxicity Potential of Bisphenol A, were included in this critique:

1. Ceccarelli, I., Della Seta, D., Fiorenzani, P., Farabollini, F., and Aloisi, A.M. (2007) Estrogenic chemicals at puberty change ERα in the hypothalamus of male and female rats. Neurotoxicol. Teratol. 29:108-115.

2. Della Seta, D., Minder, I., Dessì-Fulgheri, F., and Farabollini, F. (2005) Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain Res. Bull. 65:255-260.

3. Della Seta, D., Minder, I., Belloni, V., Aloisi, A.M., Dessì-Fulgheri, F., and Farabollini, F. (2006) Pubertal exposure to estrogenic chemicals affects behavior in juvenile and adult male rats. Horm. Behav. 50:301-307.

4. Facciolo, R.M., Madeo, M., Alò, R., Canonaco, M., and Dessi-Fulgheri, F. (2005) Neurobiological effects of bisphenol A may be mediated by somatostatin subtype 3 receptors in some regions of the developing brain. Toxicol. Sci. 88:477-484.

5. Fujimoto, T., Kubo, K., and Aou, S. (2006) Prenatal exposure to bisphenol A impairs sexual differentiation of exploratory behavior and increases depression-like behavior in rats. Brain Res. 1068:49-55.

6. Funabashi, T., Kawaguchi, M., Furuta, M., Fukushima, A., and Kimura, F. (2004) Exposure to bisphenol A during gestation and lactation causes loss of sex difference in corticotrophin-releasing hormone-immunoreactive neurons in the bed nucleus of the stria terminalis of rats. PNEC 29:475-485.

7. Gioiosa, L., Fissore, E., Ghirardelle, G., Parmigiani, S., and Palanza, P. (2007) Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. Horm. Behav. 52:307-316.

8. Honma, T., Miyagawa, M., Suda, M., Wang, R.-S., Kobayashi, K., and Sekiguchi, S. (2006) Effects of perinatal exposure to bisphenol A on brain neurotransmitters in female rat offspring. Indus. Health 44:510-524.

9. Ishido, M., Morita, M., Oka, S., Masuo, Y. (2005) Alteration of gene expression of G protein-coupled receptors in endocrine disruptors-caused hyperactive rats. Reg. Peptides 126:145-153.

10. Ishido, M., Yomemoto, J., Morita, M. (2007) Mesencephalic neurodegeneration in the orally administered bisphenol A-caused hyperactive rats. Toxicol. Lett. 173:66-72.

11. Kawai, K., Murakami, S., Senba, E., Yamanaka, T., Fujiwara, Y., Arimura, C., Nozaki, T., Takii, M., and Kubo, C. (2007) Changes in estrogen receptors α and β expression in the brain of mice exposed prenatally to bisphenol A. Reg. Toxicol. Pharmacol. 47:166-170.

12. Laviola, G., Gioiosa, L., Adriani, W., Palanza, P. (2005) D-Amphetamine-related reinforcing effects are reduced in mice exposed prenatally to estrogenic endocrine disruptors. Brain Res. Bull. 65:235-240.

13. MacLusky, N., Hajszan, T., Leranth, C. (2005) The environmental estrogen bisphenol A inhibits estradiol-induced hippocampal synaptogenesis. Environ. Health Persp. 113:675-679.

14. Masuo, Y., Morita, M., Oka, S., and Ishido, M. (2004a) Motor hyperactivity caused by a deficit in dopaminergic neurons and the effects of endocrine disruptors: a study inspired by the physiological roles of PACAP in brain. Reg. Peptides 123:225-234.

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16. Mizuo, K., Narita, M., Miyagawa, K., Narita, M., Okuno, E., and Suzuki, T. (2004) Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice. Neurosci. Lett. 356:95-98.

17. Nakamura, K., Itoh, K., Yaoi, T., Fujiwara, Y., Sugimoto, T., and Fushiki, S. (2006) Murine neocortical histogenesis is perturbed by prenatal exposure to low doses of bisphenol A. J. Neurosci. Res. 84:1194-1205.

18. Nakamura, K., Itoh, K., Sugimoto, T., and Fushiki, S. (2007) Prenatal exposure to bisphenol A affects adult murine neocortical structure. Neurosci. Lett. 420:100-105.

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20. Narita, M., Miyagawa, K., Mizuo, K., Yoshida, T., and Suzuki, T. (2007) Changes in central dopaminergic systems and morphine reward by prenatal and neonatal exposure to bisphenol-A in mice: evidence for the importance of exposure period. Addict. Biol. 12:167-172.

21. Negishi, T., Kawasake, K., Suzaki, S., Maeda, H., Ishii, Y., Kyuwa, S., Kuroda, Y., and Yoshikawa, Y. (2004) Behavioral alteration in response to fear-provoking stimuli and tranylcypromine induced by perinatal exposure to bisphenol A and nonylphenol in male rats. Environ. Health Perspect. 112:1159-1164.

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23. Nishizawa, H., Imanishi, S., and Manabe, N. (2005b) Effects of exposure in utero to bisphenol A on the expression of aryl hydrocarbon receptor, related factors, and xenobiotic metabolizing enzymes in murine embryos. J. Reprod. Dev. 51:593-605.

24. Patisaul, H.B., Fortino, A.E., and Polston, E.K. (2006) Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV. Neurotoxicol. Teratol. 28:111-118.

25. Patisaul, H.B., Fortino, A.E., and Polston, E.K. (2007) Differential disruption of nuclear volume and neuronal phenotype in the preoptic area by neonatal exposure to genistein and bisphenol-A. Neurotoxicol. 28:1-12.

26. Porrini, S., Belloni, V., Della Seta, D., Farabollini, F., Giannelli, G., and Dessì-Fulgheri, F. (2005) Early exposure to a low dose of bisphenol A affects socio-sexual behavior of juvenile female rats. Brain Res. Bull. 65:261-266.

27. Razzoli, M., Valsecchi, P., and Palanza P. (2005) Chronic exposure to low doses bisphenol A interferes with pair-bonding and exploration in female Mongolian gerbils. Brain Res. Bull. 65:249-254.

28. Rubin, B.S., Lenkowski, J.R., Schaeberle, C.M., Vandenberg, L.N., Ronsheim, P.M., and Soto, A.M. (2006) Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of bisphenol A. Endocrinol. 147:3681-3691.

29. Ryan, B.C. (2005) Developmental exposure to environmental estrogens alters adult behavior in female rodents. Doctoral thesis, North Carolina State University.

30. Ryan, B.C., Howdeshell, K.L., and Gray, L.E., Jr. (2006) Sexually dimorphic lordosis behavior is more sensitive to disruption by developmental exposure to ethinyl estradiol than is reproductive morphology in the Long Evans female rat. Biol. Reprod. (special issue) 134-135.

31. Ryan, B.C. and Vandenbergh, J.G. (2006) Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. Horm. Behav. 50:85-93.

32. Shikimi, H., Sakamoto, H., Mezaki, Y., Ukena, K., and Tsutsui, K. (2004) Dendritic growth in response to environmental estrogens in the developing Purkinje cell in rats. Neurosci. Lett. 364:114-118.

33. Tando, S., Itoh, K., Yaoi, T., Ikeda, J., Fujiwara, Y., and Fushiki, S. (2007) Effects of pre-and neonatal exposure to bisphenol A on murine brain development. Brain Develop. 29:352-356.

34. Xu, X., Liu, Y., Sadamatsu, M., Tsutsumi, S., Akaike, M., Ushijima, H., and Kato, N. (2007) Perinatal bisphenol A affects the behavior and SRC-1 expression of male pups but does not influence on the thyroid hormone receptors and its responsive gene. Neurosci. Res. 58:149-155.

35. Zoeller, R.T., Bansal, R., Parris, C. (2005) Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist *in vitro*, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinol. 146:607-612.

Ceccartelli et al (2007)

Scientific Merit:

Positive Features:

1. Oral route of administration was used.
2. A positive control (17-ethinylestradiol; EE) was included.
3. Both sexes of experimental animals were assessed for treatment related effects at two different ages after dosing (postnatal days 37 and 90).
4. Defined procedures and criteria were used for identifying and quantifying the number of estrogen receptor-alpha (ER-α) neurons in three prominent hypothalamic nuclei with the highest estrogen receptors (i.e., arcuate nucleus (ARC), ventromedial nucleus (VMH) and medial preoptic area (MPA)). Included in these procedures was the use of an investigator to count the number of ER-α immunoreactive cells, who did not know (i.e. was 'blinded to') the sex or treatment conditions of the subject animals. In addition, concomitant analyses of hormonal blood levels (testosterone and estradiol) were included. These measured endpoints represent a part of the neuronal and hormonal environment associated with sexual development and reproductive function.

Negative Features and Issues Impacting Interpretability:

1. Only a single dose of Bisphenol A (BPA) (40 μg/kg/day) was used with no specific rationale given for selection of this particular dose. And only a single dose of the positive control (0.4 μg/kg/day) was used. The absence of dose response information limits the ability to interpret the significance of the reported treatment effects. In addition, a no-effect treatment level cannot be determined.
2. The assignment of animals for treatment is not adequately explained. It is not clear whether any of the animals within a treatment group were littermates. After weaning, animals were housed in groups of 4 males and 4 females and remained in those groups during the period of dosing from PND 23 to PND 30. But it is not clear whether all animals in each group were assigned to the same treatment or to different treatments; if the latter, cross contamination of treatment groups is possible (e.g., through litter bedding or feces). It is also not clear whether any of the animals within a treatment group were littermates. If littermates were in the same treatment group, this could have introduced an unintentional bias since the authors did not appear to use the litter as the statistical unit.
3. The nature of the food given to the animals was not described. Since rat food may contain certain levels of estrogenic chemicals (phytoestrogens) as contaminants, the animals in this study may have been exposed inadvertently to higher background levels of estrogenic type compounds. Also, the type of caging during gestation/lactation and during the period of dosing was not identified. If polycarbonate caging was used, the animals could have been exposed to elevated background levels of BPA since BPA is known to leach from polycarbonates. Plexiglas cages were stated as being used after dosing.

4. Two significant changes in the housing environment were made immediately after dosing, which could have had uncontrolled confounding effects on the study. Animals were dosed from PND 23 to PND 30 with both males and females being caged together under normal lighting conditions. On the day immediately after completion of dosing (i.e., PND 31): (1) males and females were separated and housed in single sex cages and (2) all animals were placed under a reversed light-dark cycle. Such an abrupt change in social conditions and light cycle could have affected the animals' hormonal, physiological, and/or behavioral states with unknown interactive effects on the experimental treatments. This may potentially confound interpretation of the study data.
5. The ANOVA results for the ER-α neuron cell counts in the arcuate nucleus of the hypothalamus at PND 37 are presented as ($F(1,89)=95.6$, $p<0.0001$) for the Sex factor and ($F(2,89)=10.9$, $p<0.0001$) for Treatment. At PND 90 the Sex factor is ($F(1, 96)=4.46$, $p<0.03$). The degrees of freedom of 89, 89 and 96, respectively, appear incorrectly elevated. The statistical analyses of this paper should be evaluated by a statistician.

Relevance to Humans:

The primary endpoints measured in this study (i.e., brain ER-α neuronal counts, serum levels of testosterone and estradiol, and estradiol/testosterone ratios) are basic elements involved in mammalian reproductive physiology, including humans. However, in the absence of appropriate dose-response information and valid correlative behavioral data, it is difficult to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the singular dose of 40 µg BPA/kg/day, it is questionable whether the designation of LOAEL should be applied since the significance of the findings, including whether they are "adverse", is unclear and since this study was not designed as a safety assessment study and used only one treatment level.

The nature of this study appears to be focused more on hypothesis generation regarding BPA's potential biological effects and the development of appropriate methods/study designs, and less on safety assessment. As such, however, this study did report several experimental findings associated with BPA exposure in juvenile animals, the more salient effects being a selective increase in brain ER-α neuronal cells and decreased levels of serum testosterone in the 37 day old animals. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols that will also provide appropriate dose-response information to estimate applicable LOAELs and NOAELs for BPA.

(return to list)

Della Seta et al (2005)

Scientific Merit:

Positive Features:

1. Females (dams) were randomly assigned to treatment groups.
2. Oral route of dosing was used.
3. The behavioral indices of maternal behavior were clearly defined and tested at two periods during lactation, PND 3/4 and PND 8/9,
4. Procedurally, on each test day, the whole litter was removed from the dam's cage. On the first test day, 4 pups (same sex) were placed in the cage with the mother; on the second day, the 4 pups of opposite sex were placed in the cage with the dam. A counterbalanced procedure was used to avoid any confounding effects of order of presentation.

Negative Features and Issues Impacting Interpretability:

1. Only a single dose of Bisphenol A (BPA) (40 µg/kg/day) was used with no specific rationale given for selection of this particular dose. The absence of dose-response information limits the ability to interpret the significance of the reported treatment effects. In addition, a no-effect treatment level cannot be determined.
2. A positive control treatment was not used. Without a positive control, it is not possible to determine the sensitivity of the animal model or to gauge the sensitivity of the test endpoints (maternal behaviors) to estrogen treatment.
3. The nature of the food given to the animals was not described. Since rat food may contain certain chemicals with estrogenic activity (e.g., phytoestrogens) as contaminants, the animals in this study may have been exposed inadvertently to higher background levels of estrogenic type compounds. Also, the type of caging used in this study was not identified. If polycarbonate caging was used, the animals could have been exposed to additional elevated background levels of BPA, since BPA is known to leach from polycarbonates.
4. Of the 40 females mated, 17 were randomly assigned to the BPA treatment group and 23 to the oil control group. It is puzzling why equal numbers of dams were not assigned to each test group.
5. There were a number of dams in both test groups that did not deliver litters, but with an apparent greater number of non-deliveries in the BPA group compared with the oil control group. Approximately 22% of control dams failed to deliver litters, while 47% of the BPA treated dams did not deliver litters. This was not analyzed in the study and not discussed by the authors. While this lower percent of deliveries in BPA relative to control dams may be an effect of BPA treatment, this type of toxicity, particularly at such a low dose level, has not been associated with BPA exposure. Alternatively, these low birth rates may reflect some type of health problem with the experimental animals or the result of inadvertent exposure of the animals to a contaminant.

Considering the low birth rates with an unknown cause, the interpretability of the study in general and of the findings regarding maternal behavior in particular is questionable.

6. In the Discussion the authors overstate the significant findings of this study. They conclude that "On the whole, this study shows that maternal behavior is affected, both its active and passive components, by oral treatment with BPA during pregnancy and lactation." The singular significant effect of BPA treatment in the dams was an overall significant ($p<0.05$) small reduction in "licking-grooming duration" (not frequency) but only non-significant trends in several other measures (perhaps at higher dose levels these trends could possibly be significant). The biological relevance of these minimal changes is questionable. Pup body weight (growth) measured at several time points during lactation was unaffected.

Relevance to Humans:

The behavioral measures of maternal (dam) behavior used in this study have no clear relevance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the experimental design limitations and questions related to this study, particularly regarding the issue of inexplicable low birth rates, the findings from this study are not interpretable. Consequently, this study is not useable for supporting a food additive regulatory decision on BPA. The nature of this research study appears to be focused more on hypothesis generation regarding BPA's potential biological effects and the development of appropriate methods/study designs, and less on safety assessment. In addition, although minimal treatment effects were reported in this study at the singular dose of 40 µg BPA/kg/day, it is the opinion of this reviewer that the findings do not appear to be biologically relevant and consequently, it is questionable whether the designation of LOAEL should be applied to the dose level used in this study.

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Della Seta et al (2006)

Scientific Merit:

Positive Features:

1. Experimental rationale for studying both short-term effects of treatment on juvenile behavior and the long-term effects on sexual behavior of adults and relating those to steroid hormone levels was clearly explained.
2. Oral route of administering test compound was used.
3. A positive control (17-ethinylestradiol; EE) was included.
4. The authors stated reason for selection of the BPA dose was that it was in the range of environmental exposure.

Negative Features and Issues Impacting Interpretability:
(*Note that comments #1 - 4 below are the same as for Ceccarelli et al (2007) since both studies appeared to use identical pretest housing and dosing procedures*)

1. Only a single dose of Bisphenol A (BPA) (40 µg/kg/day) was used and only a single dose of the positive control (0.4 µg/kg/day) was used. The absence of dose-response information limits the ability to interpret the significance of the reported treatment effects. In addition, a no-effect treatment level cannot be determined.
2. The assignment of animals for treatment is not adequately explained. It is not clear whether any of the animals within a treatment group were littermates. After weaning, animals were housed in groups of 4 males and 4 females and remained in those groups during the period of dosing from PND 23 to PND 30. But it is not clear whether all animals in each group were assigned to the same treatment or to different treatments; if the latter, cross contamination of treatment groups is possible (e.g., through litter bedding or feces). It is also not clear whether any of the animals within a treatment group were littermates. If littermates were in the same treatment group, this could have introduced an unintentional bias since the authors did not appear to use the litter as the statistical unit.
3. The nature of the food given to the animals was not described. Since rat food may contain certain levels of estrogenic chemicals (phytoestrogens) as contaminants, the animals in this study may have been exposed inadvertently to higher background levels of estrogenic type compounds. Also, the type of caging during gestation/lactation and during the period of dosing was not identified. If polycarbonate caging was used, the animals could have been exposed to elevated background levels of BPA since BPA is known to leach from polycarbonates. Plexiglas cages were stated as being used after dosing.
4. Two significant changes in the housing environment were made immediately after dosing, which could have had uncontrolled confounding effects on the study. Animals were dosed from PND 23 to PND 30 with both males and females being caged together under normal lighting conditions. On the day immediately after completion of dosing (i.e., PND 31): (1) males and females were separated and housed in single sex cages and (2) all animals were placed under a reversed light-dark cycle. Such an abrupt change in social conditions and light cycle could have affected the animals' hormonal/ physiological/ behavioral state with unknown interactive effects on the experimental treatments. This may potentially confound interpretation of the study data.
5. In the test of adult socio-sexual behavior there are several procedural details that are not addressed in the paper. Were the same or different "receptive stimulus female(s)" used for each male or for the three treatment groups (oil, BPA, EE)? What criteria were used to identify a female as "receptive"? Was the order of testing counterbalanced across treatment groups? Was the same investigator used to score the animals behaviors and was the observer blind to treatment? These procedural details are not trivial in behavioral testing,

particularly when scoring the behavior has a subjective component. If not managed properly, unintentional bias may result.

6. In the procedure for testing adult socio-sexual behavior, the session for each animal was to be "stopped at the end of the first refractory period (time from ejaculation to the next mount) or after 30 min if the animal did not ejaculate". However, there were unexpected high numbers of rats in all three treatment groups that were not very active sexually. The numbers (%) of animals achieving ejaculation within 30 min were: Oil, n=7/12 (58%); BPA, n=4/12 (33%); and EE, n=9/12 (75%). A statistical analysis of these data was not presented. In order to continue with testing sexually active animals and reporting their data, the basis for considering animals to be sexually active was changed to those animals having "at least 2 mounts". In the Results section of this paper, the authors suggested that this low number of animals achieving ejaculation was "probably due to the lack of sexual experience". However, in the Discussion, the authors misleadingly appeared to suggest rather that this was a BPA (as well as an EE) treatment related effect stating that "The few effects found with the BPA treatment were in the same direction of EE: in particular, the small proportion of BPA males that reached ejaculation within 30 min and the reduced latency to intromission." The authors did not discuss the likelihood of other possible explanations for the low sexual activity of the test animals which in fact occurred in all three treatment groups.
7. In the Discussion, the authors state that "we find here an altered pattern of sexual behavior at adulthood as a permanent effect of pubertal exposure to EE and BPA…". While this is basically an accurate statement for EE, it is an overstatement to say that BPA altered the pattern of sexual behavior. Only one of the six measures of adult socio-sexual behavior was significantly affected by BPA, that being a decrease in intromission latency; six measures were affected by EE. BPA treatment did, however, result in significant reductions in serum testosterone levels at both the PND 37 and 105 age periods.

Relevance to Humans:

The measures of juvenile behavior and adult socio-sexual behavior of the rat represent species-specific behavioral endpoints that may serve as biomarkers of possible treatment related changes in development and sexual reproduction, but have no direct relevance to humans. The steroids measured in this study (i.e. testosterone and estradiol) are basic elements involved in mammalian reproductive physiology, including humans. However, in the absence of appropriate dose-response information and valid correlative behavioral data it is difficult to interpret the biological significance of any of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design and the inexplicable low proportion of adult animals achieving ejaculation within 30 min of testing, this study has little, if any, direct safety assessment utility for

supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the singular dose of 40 µg BPA/kg/day, it is questionable whether the designation of LOAEL should be applied since the significance of the findings, including whether they are "adverse", is unclear and since this study was not designed as a safety assessment study and used only one treatment level.

This study appears to be focused on hypothesis testing, specifically to test whether exposure to environmental xenoestrogens, such as BPA, at early puberty could affect the development of socio-sexual behavior in the male rat, and the development of appropriate methods/study designs, specifically the study of species-specific behavioral end-points as biomarkers of possible xenoestrogen influences on development and sexual differentiation. As such, however, this study did report several experimental findings associated with BPA exposure in juvenile animals, the more salient effect being a significant decrease in serum testosterone levels in both the 37 day old juveniles and the 105 day old adults. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols that will also provide appropriate dose-response information to estimate the applicable LOAEL and NOAEL for BPA.

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Facciolo et al (2005)

Scientific Merit:

Positive Features:

1. Oral route of administering test compound was used.
2. Two dose levels of BPA were used: 40 µg/kg/day and 400 µg/kg/day
3. Dose selection was based on a previous report showing neuroanatomical morphological variations and on the EPA calculated reference dose for BPA.

Negative Features and Issues Impacting Interpretability:

1. A positive control estrogenic agent was not used. This precludes verifying the basic estrogen sensitivity of the experimental model and endpoints used in this study. In the absence of a positive control, it is difficult to attribute any of the findings for BPA on somatostatin (sst_3) mRNA expression pattern to its estrogenic actions.
2. Only female (not male) offspring from treated dams were tested.
3. There was no corresponding assessment of behavioral function with which to correlate the BPA effects on sst_3 mRNA expression patterns in the female brain.
4. The nature of the food given to the animals was not described. Since rat food may contain certain levels of estrogenic chemicals (phytoestrogens) as contaminants, the animals in this study may have been exposed inadvertently to higher background levels of estrogenic type compounds.

Relevance to Humans:

The neurochemical systems (i.e., somatostatin receptor subtype 3 mRNA [sst_3-mRNA] and the α-$GABA_A$ receptor subunit) involved in this study and their regional brain distribution are basic elements whose exact involvement in development and mammalian reproductive physiology is still being investigated. Until a better understanding of the role of these systems in the development and function of the mammalian reproductive system is better understood, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

This study appears to be focused on mechanistic hypothesis testing, specifically to determine the effect of placental and lactational exposure to environmental xenoestrogens, such as BPA, on sst_3 mRNA expression patterns in the female rat brain, to understand whether this requires involvement of the α$GABA_A$ receptor subunit, and to establish a regional specificity for this effect. Since the significance of these types of effects is not completely understood, this study has no direct safety assessment utility for supporting a food additive regulatory decision on BPA. Also, the designation of an LOAEL based on these study results may be inappropriate since the significance of the findings, including whether they are "adverse", is unclear and since this study was not designed as a safety assessment study.

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Fujimoto et al (2006)

Scientific Merit:

Positive Features:

1. Oral route of administration was used (drinking water).
2. Well-described standard procedures were used for behavioral testing.

Negative Features and Issues Impacting Interpretability:

1. The number of dams/litters per treatment group used in this study (n=6) is lower than typically used for acceptable experimental studies.
2. Appropriate statistical analyses of developmental data use litter as the statistical unit in consideration of litter effects. It is apparent that all of the behavioral data in this study were analyzed using the individual offspring as the statistical unit and the litter effect was not considered. This inappropriately inflates the group N values and artificially elevates the sensitivity of the statistical comparisons. Consequently, the significant findings in this study are questionable.
3. Only a single concentration (0.1 ppm) of BPA in the drinking water was used. Consequently, dose-response information cannot be obtained nor can a NOAEL be determined. Also, the authors state that the estimated daily intake

in this study was 15 µg/kg/day, but there was no explanation as to how this estimate was derived and no range was provided.

4. Effects on the offspring were confounded by potential direct exposure of the pups to the treated drinking water as they started drinking on their own late in lactation.
5. A positive control estrogenic agent was not used. This precludes verifying the estrogen sensitivity of the experimental model and endpoints used in this study.
6. There were no corresponding measures of neurohistological, neurochemical or hormonal changes to correlate with the behavioral findings and assess their biological relevance.
7. It is not clear whether the food given to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens), nor did the authors describe any attempts to determine the chemical purity of the tap water used. Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a component in polycarbonate materials.

Relevance to Humans:

The relevance of valid animal behavioral testing to humans is inherent in its use as a biomarker of change to the functional integrity of the mammalian nervous system. However, in the absence of dose-response information, appropriate statistical analyses, and valid correlative morphochemical or hormonal data, it is difficult to interpret the biological significance of any of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the experimental design limitations and questions related to this study, it is difficult to interpret the significance of the findings from this study. Consequently, this study is not useable for supporting a food additive regulatory decision on BPA and the assignation of an LOAEL is not appropriate.

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Funabashi et al (2004)

Scientific Merit:

Positive Features:

1. Oral route of administration was used (drinking water).
2. Both sexes of experimental animals were assessed for treatment related effects as adults.
3. The numbers of corticotrophin-releasing hormone (CRH) neurons in well-defined areas were counted by visual inspection of an investigator unaware of (i.e., 'blinded to') the experimental group.

4. Efforts were made to assure uniform reproductive state of females at the time of brain tissue collection.

Negative Features and Issues Impacting Interpretability:

1. Only a single treatment level of Bisphenol A (BPA) (10 mg/L) dissolved in 0.1% ethanol in the drinking water was used and no specific rationale given for selection of this particular dose. The absence of dose response information limits the ability to interpret the significance of the reported treatment effects. In addition, a no-effect treatment level cannot be determined.
2. An incomplete set of controls was used in this study. Only one control was used, an ethanol vehicle control, in which a group of dams was given 0.1 % ethanol in their drinking water. It would have been important to have had an additional plain water control (i.e., no ethanol) to determine whether the ethanol itself was eliciting effects. Without this additional water control, possible effects of ethanol itself confound interpretation of the study results.
3. The estimated average dose of exposure to rat dams was approximately 2.5 mg/kg/day but no description was provided as to how this estimate was calculated and no range was given to indicate the variation across the gestation and lactation periods.
4. Effects on the offspring were confounded by potential direct exposure of the pups to the treated drinking water as they started drinking on their own late in lactation.
5. A positive estrogenic control treatment was not used without which it is not possible to gauge the sensitivity of the animal model and study endpoints to estrogen types of treatment.
6. There were no corresponding measures of neurochemical, hormonal or behavioral changes to correlate with the immunohistological findings and to help assess the biological significance of these findings.
7. It is not clear whether the food given to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens), nor did the authors describe any attempts to determine the chemical purity of tap water used as drinking water and to administer the test compound, BPA. Regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.
8. Although the significance to this study is not clear, a question may be raised regarding the possibility of litter effects on the endpoints measured, even in the 4 – 7 month old adult offspring. This was not mentioned by the authors and it seems unlikely that the statistical analyses, as described, took this possibility into account. This possibility should be considered in any subsequent similar studies.

Relevance to Humans:

The primary endpoints measured in this study (i.e., CRH neurons in the brain preoptic area (POA) and the bed nucleus of the stria terminalis (BST)) are basic elements involved in mammalian reproductive physiology, including humans.

However, as the authors state "the function of the BST in rats is obscure, and thus the physiological meaning of the loss of sex differences in this structure due to BPA exposure is not clear at present". Until additional relevant information is developed and appropriate dose-response information and correlative hormonal and behavioral data are provided, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design and inadequate controls, and interpretation of uncertainties, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the estimated average dose of 2.5 mg BPA/kg/day, it is questionable whether the designation of LOAEL should be applied since the significance of the findings, including whether they are "adverse", is unclear and since this study was not designed as a safety assessment study and used only one treatment level.

The nature of this research study appears to be focused more on hypothesis generation regarding BPA's potential biological effects and the development of appropriate methods/study designs, and less on safety assessment. As such, however, this study did report several suggestive findings in adult offspring associated with developmental exposure to BPA, which may serve to help identify potential sensitive endpoints. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols that will also provide appropriate dose-response information to estimate the applicable LOAEL and NOAEL for BPA.

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Gioiosa et al (2007)

Scientific Merit:

Positive Features:

1. Oral route of administration (syringe feeding) was used.
2. An acceptable number of pregnant mice per group was used (n=12-16).
3. Both male and female offspring were tested as adolescents (PND 30) and young adults (PND 70).
4. The methods/criteria for the behavioral testing (adolescents in novelty-seeking tests, and adults in free-exploratory open-field and elevated plus maze) were clearly described. Efforts were made to minimize confounding variables in the testing procedure, including cleaning of test chambers after each animal to minimize residual odor cues, training and blinding of observers to experimental groups, and using only data from adult females that were in

diestrus to minimize the influence of circulating estrogens on explorative behavior.
5. Only 1 male/female per litter were used for behavioral testing to eliminate the confound of litter effect.

Negative Features and Issues Impacting Interpretability:
1. Pregnant mice were exposed to BPA at only a single dose of 10 µg/kg/day from gestation day 11 until postnatal day 8. The absence of response measures at several dose levels limits the ability to assess the significance of single dose treatment effects. In addition, a NOAEL cannot be determined.
2. A positive estrogenic control treatment was not used. In the absence of a concomitant positive control it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to estrogenic types of treatment or to attribute the treatment effects to an estrogenic action of BPA.
3. There were no concomitant histomorphologic evaluations, hormonal analyses, or neurochemical assessments with which to correlate the treatment related behavioral effects of perinatal BPA exposure. The availability of such correlative information would have been of value in helping to assess the biological relevance of the treatment related diminished or reversal of sexually dimorphic behaviors.
4. The novelty seeking and free-exploratory open-field tasks are relatively unique. However, there were no positive controls used in the study to demonstrate the validity, sensitivity, or reliability of these latter test measures.
5. Since the magnitude of many of the male/female dimorphic behavioral endpoints in controls are relatively small, some historical information about the replicability of these male/female differences in this laboratory environment would enhance the ability to assess the biological significance of the treatment related changes in the sexually dimorphic behaviors.
6. It is known that BPA may leach from polycarbonate plastics. Since the pregnant mice in this study were housed in polycarbonate cages, it is possible that the experimental animals may have been inadvertently exposed to contaminant levels of BPA adding a potential confound to the interpretation of this study's findings. In addition, it is unknown whether the water bottles used were plastic and whether the standard mouse chow used in this study was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). The authors also did not describe whether the chemical purity of the drinking water was assessed.

Relevance to Humans:
The test paradigm in this study (i.e., the ethological assessment of changes in sexually dimorphic behaviors of adolescent and adult animals) may serve to infer changes in sexual differentiation of the mammalian brain, including humans. However, further work is needed to replicate the BPA related findings on sexually dimorphic behaviors using an appropriate experimental design and to clarify the neurobiological basis for long-term alterations from perinatal exposure to a low dose of BPA. Until such additional information is developed with appropriate

correlative morphochemical and hormonal data, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design and the possible confound of contaminant exposure to BPA, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the dose level of 10 µg BPA/kg/day, it is questionable whether the designation of LOAEL can be made with any confidence, since only one treatment level of BPA was used and since this study was not designed as a safety assessment study.

The nature of this study appears to be focused more on hypothesis testing regarding BPA's potential developmental effects on sexual differentiation of the brain and the development of sensitive biomarkers of these effects, and less on specific safety assessment. As such, however, this study did report several suggestive findings regarding the utility of sexually dimorphic behaviors in helping to identify possible subtle treatment related effects associated with developmental exposure to BPA. Efforts to confirm such possible developmental effects of BPA should utilize well-designed safety assessment animal protocols with correlative morphochemical, hormonal and behavioral endpoints and appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Honma et al (2006)

Scientific Merit:

Positive Features:

1. Oral route of administration (gavage) was used.
2. Multiple dose levels of BPA were used.
3. Both sexes of experimental offspring from treated dams were killed for brain neurotransmitter analyses at various ages through 9 weeks of age. However only females were reported in this paper; analyses of male brains are in progress and are to be reported subsequently.
4. Various brain regions were analyzed for neurotransmitters (except week 1 pups whose brains were too small for regional dissection)
5. Efforts were made to control for litter effect by selecting pups from different litters at each time period of brain analysis.

Negative Features and Issues Impacting Interpretability:

1. The numbers of litters per treatment group (n=6) and especially the numbers of offspring used for neurochemical analyses at the various age periods (n=4-6; n=1 to 10 at week one of age) are borderline for generating reliable data.
2. The statistical analyses of all data appeared to be limited to Dunnett's multiple t-tests. Due to the repetitive use of a large number of statistically "unprotected" t-tests, there is an increased probability of the occurrence of multiple false positive statistically significant findings.
3. A positive estrogenic control treatment was not used without which it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to estrogen types of treatment.
4. There were no corresponding measures of hormonal or behavioral changes to correlate with the neurotransmitter findings and to help assess the biological significance of the reported findings.
5. It is not clear whether the food (CE-2, Japan Clea, Inc) which was fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens), nor did the authors describe any attempts to determine the chemical purity of tap water used as drinking water. Regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The primary endpoints measured in this study (i.e., neurotransmitters and their metabolites) are basic elements involved in mammalian nervous system function, including humans. However, as the authors state "at present we have no data to explain the reason why the changes in monoamines and metabolites occurred in pups as well as dams". Until additional relevant information is developed and appropriate correlative hormonal and behavioral data can be provided, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design, and the interpretation uncertainties, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the dose level of 4 mg BPA/kg/day, it is questionable whether the designation of LOAEL can be made with any confidence since the significance of the findings, including whether they are "adverse", is unclear and since this study was not designed as a safety assessment study and used only one treatment level.

The nature of this research study appears to be focused more on hypothesis generation regarding BPA's potential biological effects and the development of appropriately sensitive biomarkers and study designs, and less on specific safety assessment. As such, however, this study did report several suggestive findings in the offspring associated with developmental exposure to BPA, which may serve

to help identify potential sensitive endpoints. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols that will also provide more appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Ishido et al (2005)

Scientific Merit:

Positive Features:

1. None

Negative Features and Issues Impacting Interpretability:

1. BPA was one of several "endocrine disruptors" that was intracisternally injected into 5-day old pups (87 nmol/10 µL/pup). Without dose-response information a NOAEL cannot be determined.
2. Although not specifically stated in the methods section, it is assumed that only male animals were treated and tested in this study as stated in the abstract. The authors state in the Materials and Methods that "50 male pups were born from 10 pregnant females, 5-7 of which were randomly housed and weaned…". No mention is made of female offspring being used in this study.
3. There was no information provided as to how many pups were used for the behavioral (motor activity) assessment, the DNA analyses, or the assessment of tyrosine hydroxylase immunoreactivity.
4. The assessment of motor activity was appropriately quantified and statistically analyzed. However, the results of the DNA microarray analyses were shown only as degree of increment or decrement of individual gene expression, and no quantified measures of tyrosine hydroxylase immunoreactivity (only histological pictures of brain sections) were presented. Statements were simply made describing how these endpoints were affected by intracisternal BPA treatment but no quantitative measures were presented thereby precluding statistical evaluation of these described effects.
5. A positive estrogenic control treatment was not used without which it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to estrogen types of treatment.
6. It is not clear whether the standard laboratory chow fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). Regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The primary endpoints in this study (i.e., gene expression of G-protein-coupled receptors, brain tyrosine hydroxylase immunoreactivity, and motor activity as a

behavioral index of brain function) involve basic elements in mammalian nervous system function, including humans. However, the use of intracisternal administration of BPA limits any relevant extrapolation to humans. Until additional information is developed using a more relevant route of exposure and appropriately replicated and quantified data, it is not possible to interpret the biological significance of the treatment-related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design, in the quantitation and analysis of several principle measurements, and in the use of intracisternal injection as the route of exposure, this study as presented has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment-related findings were reported in this study at the intracisternally injected level of 87 nmol BPA/pup, it is questionable whether this value should be used as a LOAEL for several reasons, including that it was the only treatment level used, that it is more relevant and common practice to identify LOAELs as mg/kg (or mg/kg/day), and that this study was not designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding BPA's potential biological effects and the development of appropriately sensitive screening systems for detection of putative endocrine disruptors, and less on specific safety assessment. As such, however, this study did report suggestive findings associated with specific effects of BPA, which may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols with a relevant route of exposure that will also provide dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Ishido et al (2007)

Scientific Merit:

Positive Features:

1. Oral route of administration (gavage) was used.
2. Efforts were included in the design of this study to include measures of behavioral change in conjunction with measures of DNA analyses, the TUNEL labeling and an assessment of tyrosine hydroxylase immunoreactivity for purposes of correlating the treatment related affects between these various endpoints.

Negative Features and Issues Impacting Interpretability:

1. A single constant treatment level of BPA was used, 600 μg/day. This treatment was stated as being "equivalent to 12-60 mg/kg". The absence of dose-response information limits the ability to interpret the significance of the reported treatment effects. In addition, a NOAEL cannot be determined.
2. Although not specifically stated in the methods section, it is assumed that only male animals were treated and tested in this study as noted in the abstract. The statement by the authors in the Materials and Methods is that "50 male pups were born from 10 pregnant females, 5-7 of which were randomly housed and weaned…". No mention is made of female offspring being used in this study.
3. The number of pups treated in the control and BPA treatment groups was not stated. The only test procedure for which there is any indication of numbers of animals tested was for activity testing in which there were 5 in the control group and 6 in the BPA group. Note that this is a bare minimum of animals for valid data, in particular involving behavioral data. But, there was no information as to how many subjects were used for the DNA analyses, the assessment of TUNEL labeling, or the assessment of tyrosine hydroxylase immunoreactivity.
4. While the assessment of motor activity was appropriately quantified, thereby allowing statistical evaluation, there were no quantitative assessments of the stated treatment effects on DNA analyses (picture of gel strip shown), TUNEL labeling (picture of brain sections shown) or tyrosine hydroxylase immunoreactivity (picture of brain sections shown). Statements were simply made that these endpoints were affected by BPA treatment but no quantitative measures were presented thereby precluding statistical evaluation of these described effects.
5. A positive estrogenic control treatment was not used without which it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to estrogen types of treatment.
6. It is not clear whether the standard laboratory chow fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The primary endpoints in this study (i.e., gene expression levels of dopamine transporter in the brain, tyrosine hydroxylase and apoptotic cells in brain, and activity as a behavioral index of brain function) involve basic elements in mammalian nervous system function, including humans. However, until additional relevant information is developed including appropriately replicated and quantified data, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design and the quantitation and analysis of several principle measurements, this study as presented has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the constant exposure level of 600 µg/pup/day, it is questionable whether this value should be used as a LOAEL for several reasons, including this was the only treatment level used, it is more relevant and common practice to identify LOAELs as mg/kg (or mg/kg/day), and this study was not designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding BPA's potential biological effects and the development of appropriately sensitive biomarkers, screening systems and study designs, and less on specific safety assessment. As such, however, this study did report several suggestive findings in association with developmental exposure to BPA, which may serve to help identify potentially sensitive and relevant endpoints that could be considered in developing well-designed animal studies to assess the safety of BPA and to provide appropriate dose-response information to estimate the applicable LOAEL and NOAEL for BPA.

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Kawai et al (2007)

Scientific Merit:

Positive Features:

1. Oral route of administration (syringe feeding) was used.
2. Experimental endpoints were obtained at several representative ages of the experimental animals (4-5, 8-9, and 12-13 weeks of age).
3. The methods/criteria for the semi-quantitative determination of immunoreactivity were well-described, including the number of animals used for each endpoint (ER-α, ER-β, 5-HT and 5-HT transporter), with n's ranging from 8 to 12.
4. Animals were housed in polypropylene cages (use of polypropylene rather than polycarbonate cages minimizes contaminant exposure to BPA in polycarbonate materials)

Negative Features and Issues Impacting Interpretability:

1. Pregnant dams were exposed to BPA at only a single dose of 2 µg/kg/day for 7 days from gestation day 11 to 17. The absence of dose response information limits the ability to interpret the significance of the reported treatment effects. In addition, a NOAEL cannot be determined.
2. Only male offspring were tested.
3. A positive estrogenic control treatment was not used. In the absence of a concomitant positive control, it is not possible to gauge the sensitivity of the

animal model and endpoints used in this study to estrogenic types of treatment.

4. There was no concomitant assessment of possible correlative effects on behavioral function or changes in sex development with which to correlate the BPA associated effects on expression of estrogen receptors α and β in the dorsal raphe nucleus. In addition, no information was given about other possible adverse effects of treatment on parturition (e.g., number of pups born live/dead, average birth weights, and pup sex ratio), postnatal body weights (growth), or developmental landmarks. These types of information would have been of use in helping to determine the biological relevance of the reported effects on expression of estrogen receptors.
5. Separate groups of male offspring were randomly selected for assessment of each of the various endpoints: measurement of serum testosterone and immunohistochemical assessment of expression of ER-α receptors, ER-β receptors, 5-HT and 5-HT transporters (n's = 8 to 18 per group). However, for each endpoint, it was not stated how many males were taken from the same litters within each treatment group (i.e., was litter effect considered).
6. The methods of sacrificing animals for the various endpoints were not stated. Certain methods of sacrificing may have confounding effects on particular measured endpoints.
7. There is no statement whether the investigator doing the immunohistochemical evaluations was aware of the group treatment.
8. The testosterone data showed a high degree of variability.
9. It is not clear whether the food fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens), nor did the authors describe any attempts to determine the chemical purity of the drinking water.

Relevance to Humans:

The primary endpoints measured in this study (i.e., activation of estrogen receptors and serum testosterone levels), are basic elements involved in mammalian sex related and nervous system functions, including humans. However, as the authors pointed out, a direct connection between ER expression and behavioral change has not been proven and future studies with a positive control and multiple doses will be necessary to clarify how a low dose of BPA affects the brain. Until additional relevant information is developed with appropriate correlative behavioral data, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the dose level of 2 µg BPA/kg/day, it is questionable whether the designation of LOAEL can be made with any confidence, since the significance of the findings, including whether they are

"adverse", is unclear and since this study was not designed as a safety assessment study and used only one treatment level.

The nature of this research study appears to be focused more on hypothesis testing regarding BPA's potential biological effects and the development of appropriately sensitive biomarkers and study designs, and less on specific safety assessment. As such, however, this study did report several suggestive findings in the offspring associated with developmental exposure to BPA, which may serve to help identify potential sensitive endpoints. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols that will also provide more appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Laviola et al (2005)

Scientific Merit:

Positive Features:

1. Oral route of administration (syringe feeding) was used.
2. An acceptable number of pregnant mice per group was used (n=10-12).
3. Both male and female offspring were tested.
4. The methods/criteria for the conditioned place preference behavioral test were clearly described. Efforts were made to minimize confounding variables in the testing procedure (e.g., testing of experimental groups was counterbalanced across time and test chambers were cleaned after each animal to minimize residual odor cues).

Negative Features and Issues Impacting Interpretability:

1. Pregnant mice were exposed to BPA at only a single dose of 10 µg/kg/day for 8 days from gestation day 11 to 18. The absence of dose response information limits the ability to interpret the significance of the reported treatment effects. In addition, a NOAEL cannot be determined.
2. A positive estrogenic control treatment was not used. In the absence of a concomitant positive control, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to estrogenic types of treatment.
3. There were no concomitant hormonal analyses or neurochemical assessments of the functional status of the dopaminergic system in the CNS with which to correlate the treatment related behavioral effects of prenatal BPA exposure. The availability of such correlative information would have been of value in helping to determine the biological relevance of the prenatal BPA effects on adult amphetamine-induced conditioned place preference.
4. The authors attribute the diminished amphetamine-induced conditioned place preference in the BPA exposed female offspring to an effect of the prenatal BPA treatment on the functional development of some component of the

central dopamine systems. However, another component of the testing conducted in this study (i.e., motor activity) which also involves dopaminergic function, was not significantly affected in the BPA exposed male or female offspring. The authors did not attempt to reconcile these two divergent results and it remains unclear what biological basis may underlie the reported decrease in amphetamine-induced conditioning.

5. It is not clear what type of caging was used (polycarbonate or some other material) or what type of food was fed to the animals and whether the food was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). The authors also did not describe whether the chemical purity of the drinking water was assessed.

Relevance to Humans:

The test paradigm in this study (i.e., the use of behavioral responses to challenge with neuroactive chemicals such as amphetamine) may provide indirect evidence of possible changes in some component of the central monoamine pathways. Monoamines are basic elements involved in mammalian nervous system function, including humans. However, as the authors pointed out, further work is needed to clarify the neural basis of the possible long-term neurobehavioral alterations from perinatal exposure to a low dose of BPA. Until additional relevant information is developed with appropriate correlative neurochemical and hormonal data, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design, and the need for clarification of the divergent findings regarding BPA's effects on amphetamine-induced changes in behaviors associated with the brain dopamine systems, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the dose level of 10 µg BPA/kg/day, it is questionable whether the designation of LOAEL can be made with any confidence, since only one treatment level of BPA was used and since this study was not designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding BPA's potential biological effects and the development of appropriately sensitive biomarkers and study designs, and less on specific safety assessment. As such, however, this study did report several suggestive findings regarding the sensitivity of pharmacologic challenge in helping to identify possible subtle treatment related effects associated with developmental exposure to BPA. Efforts to confirm possible developmental effects of BPA should utilize well-designed safety assessment animal protocols that will also provide more appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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MacLusky et al (2005)

Scientific Merit:

Positive Features:

1. No notable positive features.

Negative Features and Issues Impacting Interpretability:

1. Parenteral (subcutaneous) administration of BPA was used.
2. The number of animals in each treatment group was n=3. The authors stated that their lab has verified that the use of three animals per treatment group provides sufficient statistical power to detect effects …because of the precision obtained by analyzing large numbers of sections (histological) per animal. However, the determination of the appropriate sample size (N's) per treatment group is based not only on precision/sensitivity for detection but should also consider whether the sample size (i.e., number of test subjects per treatment group) is sufficiently large to be representative of the population (i.e., population variance). With regard to the latter, three animals is not an adequate sample size.
3. A positive control for the BPA treatment was not used. In the absence of a concomitant positive control it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
4. There were no concomitant behavioral assessments, specifically of cognitive changes, with which to correlate the treatment related effects of BPA on estradiol induced hippocampal synaptogenesis. The availability of such correlative information would have been of value in helping to determine the biological relevance of the reported effects of BPA.
5. It is not clear whether the standard laboratory chow fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). The authors did discuss the presence of estrogenic contaminants in rat chow but did not describe the nature of the food used in this study. There was also no description of whether measures were taken to provide purified drinking water to the animals. Regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The endpoint in this study (i.e., estradiol-induced hippocampal synaptogenesis) is a basic element involved in mammalian nervous system function, including humans. However, this study was conducted in ovariectomized adult females. As the authors pointed out, it is critical to determine whether the estradiol-induced hippocampal synaptogenesis is similarly affected by low doses of BPA under

(normal) conditions of sustained physiologic circulating levels of estradiol. Until such additional relevant information is developed with appropriate correlative behavioral (cognitive) functional assessments, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design, and the need for additional critical information to determine whether the reported effects of BPA on estradiol induced hippocampal synaptogenesis would be similarly affected under conditions of sustained physiological levels of estradiol, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the subcutaneous dose level of 40 µg BPA/kg/day, it is questionable whether the designation of LOAEL can be made with any confidence, since this study was not designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding BPA's potential biological effects and the development of appropriately sensitive biomarkers for use in screening putative estrogen-like "endocrine disruptors", and less on specific safety assessment. As such, however, this study did report suggestive findings associated with specific effects of BPA, which may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols that will also provide more appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Masuo et al (2004a)

Scientific Merit:

Positive Features:

1. None

Negative Features and Issues Impacting Interpretability:

1. BPA was one of several "endocrine disruptors" that was intracisternally injected into 5-day old pups (87 nmol/10 µl/pup) (n=5 to 7). The dose level was not expressed in the more common mg/kg format. Without dose response information, a NOAEL cannot be determined.
2. Only male pups were treated and tested in this study. There was no information provided as to how many pups were used for the behavioral (motor activity) assessment, the DNA analyses, or the assessment of tyrosine hydroxylase immunoreactivity.

3. The assessment of motor activity was appropriately quantified and statistically analyzed. However, the results of the DNA microarray analyses were shown as the ratio of the individual gene expression level versus that of the respective control. Statements were simply made describing how the expression of the various genes were affected by the intracisternal BPA treatment but no statistical evaluation of these described effects were conducted.
4. A positive estrogenic control treatment was not used without which it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to estrogen types of treatment.
5. It is not clear whether the standard laboratory chow fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). Also, regarding other potential sources of contaminant exposure, the purity of the tap water used as drinking water was apparently not determined, and the types of water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials. Acrylic cages were used for housing the animals.

Relevance to Humans:

The primary endpoints in this study (i.e., multiple gene expression changes and motor activity as a behavioral index of brain function) involve basic elements in mammalian nervous system function, including humans. However, until additional relevant information is developed using a more relevant route of exposure and correlative neurochemical and hormonal measurements, and appropriately replicated and quantified data are presented, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly its experimental design, the inadequate quantification and analysis of the gene arrays, and the use of intracisternal injection as the route of exposure, this study as presented has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the intracisternally injected level of 87 nmol BPA/pup, it is questionable whether this value should be used as a LOAEL for several reasons, including that it was the only treatment level use, that it is more relevant and common practice to identify LOAELs as mg/kg (or mg/kg/day), and that this study was not designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding BPA's potential biological effects and the development of appropriately sensitive screening systems for detection of putative endocrine disruptors, and less on specific safety assessment. As such, however, this study did report suggestive findings associated with specific effects of BPA, which may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to

confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols with a relevant route of exposure that will also provide more appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Masuo et al (2004b)

Scientific Merit:

Positive Features:

1. BPA was administered across an incremental range of doses to 5 day old male rat pups (0, 0.087, 0.87, 8.7, 87 nmol/pup) thereby enabling an approximation of a lowest dose effect level as well as a no observed effect level, based on the endpoint of motor activity.

Negative Features and Issues Impacting Interpretability:

1. BPA was one of several "endocrine disruptors" that was intracisternally injected into 5-day old pups. The nmol/pup dose levels were not expressed in the more common format of mg/kg. Each of the BPA treated groups consisted of n=6 pups.
2. Only male pups were dosed and tested in this study.
3. Since a positive estrogenic control treatment was not used, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to estrogenic types of chemicals.
4. The assessment of motor activity was appropriately quantified and statistically analyzed. However, the results of the DNA microarray analyses for the "endocrine disruptors" (including BPA) were compared with results from animals treated with 6-OHDA. Two tables in the paper listed the genes in which the expression was above the threshold (signal intensities differed by more than 3-fold and expression ratios of treated rats versus control that differed by more than 1.63) in rats treated with 6-OHDA. Statements were simply made describing how the expression of the various genes were affected by the intracisternal BPA treatment but no statistical evaluation of these described effects were conducted. Similarly, treatment effects on tyrosine hydroxylase immunoreactivity were verbally described (several pictures of histological sections were shown) but no quantification was presented and no statistical evaluations done.
5. No measures of monoamine brain levels (specifically, dopamine), hormone levels or other behavioral endpoints were obtained with which to correlate the reported motor activity effects of BPA, making it difficult to evaluate the biological significance of these behavioral changes.
6. It is not clear whether the standard laboratory chow fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). Also, regarding other potential sources of contaminant exposure, the purity of

the tap water used as drinking water was apparently not determined, and the types of water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials. Acrylic cages were used for housing the animals.

Relevance to Humans:

The primary endpoints in this study (i.e., multiple gene expression changes, tyrosine hydroxylase activity, and motor activity as a behavioral index of brain function) involve basic elements reflecting mammalian nervous system function, including humans. However, until additional relevant information is developed using a more relevant route of exposure and correlative neurochemical and hormonal measurements and appropriately replicated and quantified data are provided, it is not possible to interpret the biological significance of the treatment related changes reported in this study or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly its experimental design, the inadequate quantification and analysis of the gene arrays, and the use of intracisternal injection as the route of exposure, this study as presented has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although a range of treatment levels were used and findings associated with BPA exposure were reported at several of the intracisternally injected levels (0.87, 8.7 and 87 nmol BPA/pup but no effects at the 0.087 nmol BPA/pup dose level), it is questionable whether the lowest effect level (0.87 nmol/pup) should be formally designated the LOAEL or the no effect level of 0.087 nmole/pup designated the NOAEL, since it is more relevant and common practice to identify LOAEL and NOAEL as mg/kg (or mg/kg/day) and also since this experiment was not designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding mechanisms underlying motor hyperactivity as related to endocrine disruptor exposure and the development of appropriately sensitive screening systems for detection of putative endocrine disruptors, and less on specific safety assessment. As such, however, this study did report suggestive findings associated with intracisternal BPA injection (particularly hyperactivity in the adult males), which may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols with a relevant route of exposure that will also provide dose-response information to estimate more appropriately the applicable LOAEL and NOAEL for BPA.

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Mizuo et al (2004)

Scientific Merit:

Positive Features:

1. Multiple dietary levels of BPA were used in this study (i.e., 0, 0.002, 0.5 and 2.0 mg BPA/g food). BPA was given in the diet to pregnant mice from mating until weaning of pups.

Negative Features and Issues Impacting Interpretability:

1. Actual dose levels were not calculated by the study authors, but estimated dose levels were 0, 0.4, 100 and 400 mg/kg body weight/day, respectively, based on the assumption that a female mouse consumes approximately 0.2 g food/g body weight/day.
2. Effects on the offspring were confounded by potential direct exposure of the pups to the treated diet as they started eating on their own late in lactation.
3. The authors state that during the treatment with BPA, animals (assuming reference to the dams) did not show weight loss and disrupted maternal behaviors. Body weight data were not shown and there was no explanation as to how maternal behaviors were assessed to support this statement.
4. There was virtually no information provided by the authors regarding a variety of critical methods and procedural issues, including the number of pregnant mice used, the number of litters available, number of litters per treatment group, or male/female composition of the litters. There was also no information provided about possible treatment effects on parturition, numbers of live births, pup deaths, male/female ratio, birth weights, etc. Additional critical information that was not provided are the age(s) of the offspring at the time of behavioral testing and the age(s) when animals were sacrificed for biochemical analyses. A correlative issue related to the lack of information about the number of litters per group is whether multiple pups from the same litter were used for each of the various tests. In other words, was the litter effect taken into consideration or was the individual pup used as the statistical unit? Were the same offspring used for both behavioral tests? Were the same offspring used for behavioral testing and for biochemical analyses? Without these types of basic information it is not possible to assess the relevance and validity of the study's findings.
5. A positive control for the BPA treatment was not used. In the absence of a concomitant positive control it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
6. In view of the lack of information about the type of food, source of water, and type of caging used in this study, it is unknown whether the animals may have been unintentionally exposed to estrogenic contaminants. Was the food certified to minimize any contaminant estrogenic chemicals (phytoestrogens) and was the water purified? And, finally, were the cage and water bottles used in this study made from polycarbonate material which is known to contain BPA.

Relevance to Humans:

The endpoints in this study, involving μ-opioid receptor activity and behavioral responses to opiates, are relevant to mammalian nervous system function, including humans. However, until clarification can be provided regarding the procedures and methods used in this study, it is not possible to interpret the biological significance of the treatment related changes reported or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design and the absence of critical information regarding study procedures and methods, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at dietary dose levels as low as approximately 100 mg/kg/day (based on a dietary concentration of 0.5 mg BPA/g food), it is questionable whether the designation of LOAEL for this dose can be made with any confidence, since critical information regarding the procedures and methods in the study were not provided and since this study was not specifically designed as a safety assessment study. Similarly, it is questionable whether the dietary dose level of 0.4 mg BPA/kg body weight/day (based on dietary concentration of 0.002 mg BPA/g food), at which no significant treatment related effects were observed, should be designated the NOAEL.

The nature of this research study appears to be focused more on hypothesis generation regarding BPA's potential biological effects, and less on specific safety assessment. As such, however, this study did report suggestive findings associated with effects of prenatal/postnatal exposure to BPA, specifically the changes in behavioral sensitivity to morphine reflecting an influence on the development of the central dopaminergic system. This information may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols that will also provide more appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Nakamura et al (2006)

Scientific Merit:

Positive Features:

1. A complementary set of immunohistochemical analyses, morphometry with the use of bromodeoxyuridine, and quantitative RT-PCR was used to determine whether BPA treatment during gestation affects brain cell proliferation, neuronal differentiation and migration in the mouse.

Negative Features and Issues Impacting Interpretability:

1. A single dose of BPA, 20 µg/kg, was used, being injected subcutaneously into pregnant mice daily from Gestation Day 0 until each of the three days of sacrifice (GDs 12, 14 and 16). Without dose response information, interpretation of the treatment effects is difficult and a NOAEL cannot be determined.
2. Other than the statement that pregnant mice were randomly divided into two treatment groups (BPA and control), the number of pregnant mice per group (2 treatments on 3 sacrifice days) was not specified. While the number of litters per group was also not specified, it was stated that brains from 10 embryos obtained from two or more dams (litters) in every group were used for the immunohistochemistry/morphometry analyses. Assumingly then, there were 2 or more litters in each group. Since 10 embryos were used from two or more dams, it is apparent that the litter factor was not considered and that the individual embryo/fetus was used as the statistical unit. Ignoring the litter factor artificially inflates the sensitivity of the statistical analyses resulting in possible statistical false positives.
3. There was no information provided about any changes in dam body weights or other signs of toxicity.
4. A positive control for the BPA treatment was not used. In the absence of a concomitant positive control, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
5. While statistical analyses were supposed to have been carried out on the study findings, the authors simply described certain treatment related findings as being significant or not significant, but no statistical information was provided.
6. There was no information about the type of food, source of water, and type of caging used in this study, all of which may be sources of unintentional exposure to estrogenic contaminants.

Relevance to Humans:

The focus of this study on developmental factors affecting brain cell proliferation, neuronal differentiation and migration are relevant to the development of the mammalian nervous system, including humans. However, until additional information is developed to clarify the developmental role(s) of the various endpoints measured in this study, it is not possible to interpret the biological significance of the treatment related changes reported or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design including the subcutaneous administration of BPA, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision

on BPA. In addition, although treatment related findings were reported in this study at the subcutaneous dose level of 20 µg/kg/day, it is questionable whether the designation of LOAEL for this dose should be made, since a subcutaneous route of administration was used and since this study was not specifically designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis generation regarding BPA's potential developmental effects, and less on specific safety assessment. Efforts to identify any possible adverse effects of BPA should utilize well-designed safety assessment animal protocols that will also provide appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Nakamura et al (2007)

Scientific Merit:

Positive Features:

1. A longitudinal assessment of prenatal BPA effects on cytoarchitectural development and neural connections of the brain was carried out in offspring through the postnatal age of 12 weeks.
2. Male and female offspring were used in at least several of the analyses conducted.

Negative Features and Issues Impacting Interpretability:

1. Only a single dose of BPA, 20 µg/kg, was used. It was injected subcutaneously into pregnant mice daily from Gestation Day 0 until the bromodeoxyuridine (BrdU) (used in determining cell proliferation and migration) was administered on GD 12, 14 or 16. Without dose response information, interpretation of the treatment effects is difficult and a NOAEL cannot be determined.
2. The numbers of pregnant mice assigned to each experimental group (2 treatments x 3 BrdU injection days x 7 postnatal sacrifice ages) were not specified. With regard to the number of offspring per experimental group, it was stated that 10 brains obtained from two or more dams (litters) in every group were used for quantitative analyses. Yet, confusingly, it was also stated that for some measures or groups, 10 male and 10 female brains were used. The exact numbers and sexes of animals used per group are unclear. Irrespective of the numbers of animal per group, it is apparent that offspring in each group were taken from 2 or more dams (litters). Consequently, the litter effect seems to have been ignored and the individual offspring was used as the statistical unit. Ignoring the litter factor artificially inflates the sensitivity of the statistical analyses resulting in possible statistical false positives.

3. There was no information provided about whether there were any treatment related effects on dam body weights, parturition, numbers of live births, male/female ratio, birth weights, or other signs of developmental or other toxicity.
4. A positive control for the BPA treatment was not used. In the absence of a concomitant positive control, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
5. It is unclear whether the histochemical evaluations were conducted by investigators blind to treatment conditions, which would minimize unintentional bias.
6. There were no concomitant longitudinal measures of behavioral function or of neurochemical changes with which to correlate and assess the biological relevance of the treatment related changes in cortical cytoarchitecture and neural connections in the juvenile and adult brains.
7. There was no information about the type of food, source of water, or type of caging or water bottles used in this study, all of which are potential sources of unintentional exposure to estrogenic and other contaminants.

Relevance to Humans:

The focus of this study on whether perturbed neocortical histogenesis during the prenatal period of development results in changes of cortical cytoarchitecture and neural connections in adult brain is relevant to an understanding of the development of the mammalian nervous system, including humans. However, due to the use of a subcutaneous route of exposure to BPA and until additional information is developed to clarify the functional significance of the reported changes in the various endpoints measured in this study, it is not possible to interpret the biological significance of the treatment related changes or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design including the subcutaneous administration of BPA, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the subcutaneous dose level of 20 μg/kg/day, it is questionable whether the designation of LOAEL for this dose should be made, since a subcutaneous route of administration was used and since this study was not specifically designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis generation regarding BPA's potential developmental effects, and less on specific safety assessment. Efforts to identify any possible adverse effects of BPA should utilize well-designed safety assessment animal protocols that will also provide appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Narita et al (2006)

(Note that this is an extension and partial replication of a study by the same laboratory reported previously in Mizuo et al (2004), critiqued above)

Scientific Merit:

Positive Features:

1. Multiple dietary levels of BPA were used in this study (i.e., 0, 0.03, 0.3, 3.0, 500 and 2000 mg BPA/kg food). BPA was given in the diet to pregnant mice from mating until weaning of pups.

Negative Features and Issues Impacting Interpretability:

1. Actual dose levels were not reported by the study authors, but estimated dose levels were 0, 0.006, 0.06, 0.6, 100 and 400 mg/kg body weight/day, respectively, based on the assumption that a female mouse consumes approximately 0.2 g food/g body weight/day.
2. The authors state that during treatment with BPA, animals did not show weight loss or disruption of maternal behaviors. However, body weight data were not shown and there was no explanation as to how maternal behaviors were assessed to support this statement.
3. Effects on the offspring were confounded by potential direct exposure of the pups to the treated feed as they started eating on their own late in lactation.
4. The numbers of offspring used for behavioral testing ranged from n=6 to 14 for place preference conditioning and n=5 to 15 for locomotor activity. However, in the conduct of the GTPγS binding assay, the data presented are based on n=3 samples per group. This latter is an extremely low number of subjects per group and raises questions about the reliability of these data.
5. There was virtually no information provided by the authors regarding a variety of critical methods and procedural issues, including the number of pregnant mice used per treatment group, the number of litters obtained, number of litters assigned to each treatment group, or male/female composition of the litters.
6. There was no information provided about possible treatment effects on parturition, numbers of live births, pup deaths, male/female ratio, birth weights, etc.
7. A correlative issue related to the lack of information about the number of litters per group is whether multiple pups from the same litter were used for each of the various tests. In other words, was the litter effect taken into consideration or was the individual pup used as the statistical unit? Were the same or different offspring used for both behavioral tests? Were the same or different offspring used for behavioral testing and for biochemical analyses? Without these types of basic information, it is very difficult to assess the relevance and validity of the study's findings.
8. All testing was conducted on male offspring only.

9. A positive control for the BPA treatment was not used. In the absence of a concomitant positive control, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
10. There is a lack of information about the food, water and caging used in this study. Was the food certified to minimize any contaminant estrogenic chemicals (phytoestrogens) and was the water purified? Additionally, were the cages and water bottles used in this study made from polycarbonate material which is known to contain BPA? In the absence of such information, the possibility cannot be discounted that the animals could have been unintentionally exposed to estrogenic or other contaminants.

Relevance to Humans:

The endpoints in this study, involving development of the brain dopaminergic system and adult behavioral responses to opiates (as possibly related to the dopaminergic system), are relevant to mammalian nervous system function, including humans. However, until clarification can be provided regarding the procedures and methods used in this study, it is not possible to interpret the biological relevance of the treatment related changes reported or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design and the absence of critical information regarding study procedures and methods, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at dietary dose levels as low as approximately 0.06 mg/kg/day (based on dietary concentration of 0.03 mg BPA/kg food), it is questionable whether the designation of LOAEL for this dose can be made with any confidence, since critical information regarding the procedures and methods in the study were not provided and since this study was not specifically designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis generation regarding BPA's potential developmental effects, and less on specific safety assessment. As such, however, this study did report suggestive findings associated with effects of prenatal/postnatal exposure to BPA, specifically the changes in behavioral sensitivity to morphine associated with a change in the development of the central dopaminergic system and the possibility of a nonlinear (bimodal) dose response for developmental effects of BPA, at least in male offspring. This information may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to confirm such possible effects of BPA should be incorporated in well-designed safety assessment animal protocols that will also provide appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Narita et al (2007)

(Note that this is an extension and partial replication of a study by the same laboratory reported previously in Narita et al (2006), critiqued above.)

Scientific Merit:

Positive Features:

1. Adult female mice were exposed to BPA in the diet (2000 mg/kg feed) during specific developmental periods (i.e., implantation (Gestation Days 0 – 7), organogenesis (GD 7 – 14), parturition (GD 14-20) and lactation (Postnatal Days 0 – 20)). This treatment regimen was used to determine the most sensitive period(s) in prenatal and postnatal exposure to BPA in mice.

Negative Features and Issues Impacting Interpretability:

1. Only a single dietary level of BPA was used (2000 mg/kg feed). Since dose-response information was not obtained, a NOAEL cannot be determined. The actual dose level was not reported by the study authors, but an estimated dose level is 400 mg/kg body weight/day, respectively, based on the assumption that a female mouse consumes approximately 0.2 g food/g body weight/day.
2. The authors state that during treatment with BPA, animals did not show weight loss or disrupted maternal behaviors. However, dam body weight data were not shown and there was no explanation as to how maternal behaviors were assessed to support this statement. The authors did add that the pups did not show weight loss or a decrease in birth rate.
3. Effects on the offspring were confounded by potential direct exposure of the pups to the treated feed as they started eating on their own late in lactation.
4. The numbers of offspring used for behavioral testing ranged from n=6 to 16 for place preference conditioning and n=9 to 10 for locomotor activity. However, in the conduct of the GTPγS binding assay the data presented are based on n=3 samples per group. This latter is an extremely low number of subjects per group and raises questions about the reliability of these data.
5. There was virtually no information provided by the authors regarding a variety of critical methods and procedural issues, including the number of pregnant mice used per treatment group, the number of litters obtained, the number of litters assigned to each treatment group, or male/female composition of the litters.
6. A correlative issue related to the lack of information about the number of litters per group is whether multiple pups from the same litter were used for each of the various tests. In other words, was the litter effect taken into consideration or was the individual pup used as the statistical unit? Were the same or different offspring used for both behavioral tests? Were the same or different offspring used for behavioral testing and for biochemical analyses? Without these types of basic information it is very difficult to assess the relevance and validity of the study's findings.
7. Only male offspring were used for testing in this study.

8. A positive control for the BPA treatment was not used. In the absence of a concomitant positive control, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
9. There is a lack of information about the food, water and caging used in this study. Was the food certified to minimize any contaminant estrogenic chemicals (phytoestrogens) and was the water purified? Additionally, were the cages and water bottles used in this study made from polycarbonate material which is known to contain BPA? In the absence of such information, the possibility cannot be discounted that the animals could have been unintentionally exposed to estrogenic or other contaminants.

Relevance to Humans:

The endpoints in this study, involving development of the brain dopaminergic system and adult behavioral responses to opiates (as possibly related to the dopaminergic system), are relevant to mammalian nervous system function, including humans. However, until clarification can be provided regarding the procedures and methods used in this study, it is not possible to interpret the biological relevance of the treatment related changes reported or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design and the absence of critical information regarding study procedures and methods, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the only dose level used which was estimated to be 400 mg/kg/day (based on dietary concentration of 2000 mg BPA/kg food), it is not appropriate to apply the designation of LOAEL to this dose level, since this dose appeared to have been used to maximize treatment related effects based on previous study results, since critical information regarding the procedures and methods in the study were not provided, and since this study was not specifically designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis generation regarding BPA's potential developmental effects, and less on specific safety assessment. As such, however, this study did report suggestive findings with regard to possible sensitive periods of prenatal/postnatal exposure to BPA. In addition, notwithstanding the limitations as noted above in this critique, this study did appear to replicate findings from a previous study: specifically, the enhanced sensitivity in adult offspring to behavioral effects of morphine and the up-regulation of dopamine receptor function in the limbic forebrain of adult mice developmentally exposed to a high dose level of BPA. This information may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to confirm such possible effects of BPA should be incorporated in well-designed safety assessment animal protocols that will also provide appropriate dose-

response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Negishi et al (2004)

(Note that this same paper was included in the Exponent literature review of neurobehavioral effects of Bisphenol A)

Scientific Merit:

Positive Features:

1. BPA administered orally (gavage) to pregnant rats from Gestation Day 3 until Postnatal Day 20.
2. Pregnant rats were randomly assigned to treatment groups and an acceptable number of pregnant animals was used (n=10-11 per group, apparently resulting in 9-10 litters).
3. Litter was used as the statistical unit with each litter being represented by one pup for behavioral testing.
4. Behavioral test methods were well-defined.
5. Behavioral testing was supplemented with the collection of other general endpoints which enabled an assessment of whether BPA treatment resulted in any general signs of toxicity. These other endpoints included maternal body weight, parturition information, maternal organ weights at weaning (PND 21), and general development of offspring (periodic body weights and organ weights).

Negative Features and Issues Impacting Interpretability:

1. Only a single dose of BPA, 0.1 mg/kg/day, was used. Without dose response information, interpretation of the treatment effects is difficult and a NOAEL cannot be determined.
2. A positive control for the BPA treatment was not used. In the absence of a concomitant positive control, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
3. Only males were used in this study. Therefore, sex related differences in the effects of BPA were not assessed.
4. Only one male pup from each litter was used for all behavioral testing. The use of the same animal for all behavioral testing may have introduced some confounding test-test interactions (e.g., passive avoidance being followed by active avoidance in which optimum behavioral responses are exact opposites (remaining stationary in one chamber being optimum for passive avoidance and moving rapidly from one chamber to another being optimum in active avoidance)).
5. Conducting the testing of different types of behavior over at different ages in all animals (i.e., open field at 8 weeks of age, motor activity at 12 weeks, passive avoidance at 13 weeks, elevated plus maze at 14 weeks, active

avoidance at 15 weeks, and monoamine disruption test at 22-24 weeks) makes it difficult to interpret how the findings for the various behavioral tests may relate to a common developmental effect of BPA treatment. For example, the monoamine disruption test at 22-24 weeks of age indicated possible effects on monoaminergic systems but the biological significance is unknown since it is unclear how or whether this may relate to the findings or lack of findings in the behavioral tests conducted at earlier ages (8, 12, 13, 14 and 15 weeks of age).

6. The investigators did not discuss the differential findings in the monoamine disruption test which may indicate a highly specific effect of BPA on the monoaminergic system. BPA treatment prevented the tranylcypromine-induced increase in locomotor (horizontal) activity but BPA had no suppressant affect on the tranylcypromine-induced decrease in rearing behavior.
7. There were no concomitant neurochemical or endocrine measures with which to correlate and assess the biological relevance of the few treatment related findings in behavioral testing.
8. There was no information about the type of food, source of water, and type of caging or water bottles used in this study, all of which are potential sources of unintentional exposure to estrogenic and other contaminants.

Relevance to Humans:

The focus of this study on changes in primary behavioral functions is a relevant assessment of the development of the mammalian nervous system, including humans. However, until additional information is developed to clarify the biological relevance of the few reported findings in this study, it is not possible to interpret the biological significance of the treatment related changes or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at an oral dose of 0.1 mg/kg/day, it is questionable whether the designation of LOAEL for this dose should be made, since this was the only dose level used and since this study was not specifically designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding the scope of BPA's potential developmental behavioral effects and procedures for their detection (e.g., pharmacologic challenge), and less on specific safety assessment. As such, this study did report suggestive findings associated with specific effects of BPA, which may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to confirm such possible effects of BPA should utilize well-designed safety assessment animal protocols that will also

provide more appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Nishizawa et al (2005a)

Scientific Merit:

Positive Features:

1. BPA was administered orally (gavage?) to separate groups of pregnant mice, one group exposed from Gestation Day 6 to 13 and the other from GD 6 to 17.
2. A range of dose levels was used, specifically 0, 0.02, 2, 200 and 20,000 µg/kg/day (= 0, 0.00002, 0.002, 0.2 and 20 mg/kg/day).
3. An ample number of pregnant animals were treated (i.e., n=12 per group).
4. Analyses were carried out on male and female embryos. Sex was determined by assaying embryonic liver DNA samples for the Y chromosome.

Negative Features and Issues Impacting Interpretability:

1. BPA was administered orally, but there is no clear designation as to the mode of oral administration (i.e., gavage, diet, etc.).
2. A positive control for the BPA treatment was not used. In the absence of a concomitant positive control, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
3. Although there were 12 pregnant females in each treatment group, there is no information about the number of embryos used for each assay per group.
4. Also, it is not known whether all of the embryos from the same dam were used for the same analysis, in other words whether the dam (litter) or individual embryo was used as the statistical unit. It would have been more appropriate to use the dam as the statistical unit.
5. There were no concomitant neurochemical or endocrine measures from the dams or morphochemical assessment of the embryos with which to correlate the treatment related findings in the receptor m-RNA expression. Also, since none of the pregnant animals were allowed to litter, there were no neurochemical, endocrine or behavioral/functional postnatal measures in the offspring to assess the biological relevance of the treatment related changes in the receptor m-RNA expression.
6. There was no information about whether the standard diet fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens) or whether the purity of the tap water was determined. Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The focus of this study on changes in expression of aryl hydrocarbon receptor, retinoic acid receptor α and retinoid X receptor α (key factors in embryogenesis-regulating receptors and nuclear receptor-dependent signal transduction) is relevant to the mammalian embryonic development, including humans. However, until additional information is developed to clarify the biological relevance in the animal model of the types of receptor m-RNA expression changes reported in this study, it is not possible to interpret or extrapolate their biological relevance or significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of interpretation of the effects reported in this study and to some uncertainties in particular experimental design issues, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at 0.02 µg/kg/day, the lowest oral dose used in this study, it is questionable whether the designation of LOAEL for this dose should be made, since this study was not specifically designed as a safety assessment study. A no-effect level could not be determined in this study.

The nature of this research study appears to be focused on the study of changes in expression of m-RNA for AhR, RARα and RXRα in the mammalian embryo and somewhat on hypothesis testing regarding the scope of BPA's potential effects on these key factors in embryogenesis, but was apparently not focused specifically on the safety assessment of BPA. As such, however, this study did report suggestive findings which may contribute to an assessment of the potential effects of BPA on embryogenesis. Efforts to determine effects of BPA on embryogenesis should utilize well-designed safety assessment animal protocols that will also provide correlative morphochemical, endocrine and behavioral/functional information to assess the biological relevance of any findings.

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Nishizawa et al (2005b)

Scientific Merit:

Positive Features:

1. BPA was administered orally (gavage?) to separate groups of pregnant mice, one group exposed from GD 6 to 13 and the other from GD 6 to 17.
2. A range of dose levels was used, specifically 0, 0.02, 2. 200 and 20,000 µg/kg/day (= 0, 0.00002, 0.002, 0.2 and 20 mg/kg/day).
3. A positive control (17 β-estradiol) for the BPA treatment was used.
4. An ample number of pregnant animals were dosed, i.e. n=12 per group.
5. Analyses were carried out on male and female embryos (except for embryonic liver protein levels of CYP1A1 and GST in which only one sex was used).

Sex was determined by assaying embryonic liver DNA samples for the Y chromosome.

Negative Features and Issues Impacting Interpretability:

1. BPA was administered orally, but there is no clear designation as to the mode of oral administration (i.e., gavage, diet, etc.).
2. Although there were 12 pregnant females in each treatment group, there is no information about the number of male/female embryos used for each assay per group.
3. Also, it is not known whether all of the embryos from the same dam were used for the same analysis, in other words whether the dam (litter) or individual embryo was used as the statistical unit. It would have been more appropriate to use the dam as the statistical unit.
4. In the analysis of embryonic livers for protein levels of CYP1A1 and GST only one set of data was provided representing either male or female embryos but it was not specified which sex (male or female) was used.
5. There were no concomitant neurochemical or endocrine measures from the dams or morphochemical assessment of the embryo brains with which to correlate the treatment related findings in the cerebrum and cerebellum receptor m-RNA expression. Also, since none of the pregnant animals were allowed to litter, there were no neurochemical, endocrine or behavioral/functional postnatal measures in the offspring to assess the biological relevance of the treatment related changes in the receptor m-RNA expression.
6. There appeared to be some attempt to assess the biological significance of the changes in brain and gonad expression of CYP1A1 and GST m-RNA. Embryonic liver protein levels of CYP1A1 and GST were measured. However, there wasn't a clear correlation between the BPA or E2 induced changes in liver protein levels and the BPA or E2 induced changes in brain/gonad m-RNA expression.
7. There was no information about whether the standard diet fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens) or whether the purity of the tap water was determined. Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.
8. Finally, it should be noted that, while the data on expression of AhR m-RNA was presented in this paper as being developed in the experiment described in this paper, the results for the AhR m-RNA expression (as presented in Figure 1 and described in the Results section) were virtually identical to the data on AhR m-RNA presented in a previous publication by this laboratory (Nishizawa et al, 2005a which was critiqued above). The only difference between the two graphs was the addition of E2 positive control data in the present paper. Even the text description of the data in the Results of the present paper was very similar to that in the previous paper. The description of

the AhR m-RNA data in the present study did not even mention the E2 positive control data endpoint.

Relevance to Humans:

The focus of this study on changes in expression of AhR m-RNA and related factors and AhR-mediated drug-metabolic enzymes (CYP1A1 and GST) which are key factors in mammalian embryonic development, is relevant to humans. However, until additional information is developed to clarify the biological relevance in the animal model of the BPA related changes in receptor m-RNA expression as reported in this study, it is not possible to interpret or extrapolate the biological relevance or significance of these changes to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations in interpretation of the effects reported in this study and to some uncertainties with regard to particular experimental design issues, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at 0.02 µg/kg/day, the lowest oral dose used in this study, it is questionable whether the designation of LOAEL for this dose should be made, since this study was not specifically designed as a safety assessment study. A no-effect level could not be determined in this study.

The nature of this research study appears to be focused on the study of changes in expression of m-RNA for AhR and related factors and AhR-mediated drug-metabolic enzymes (CYP1A1 and GST) in the mammalian embryo and on hypothesis testing regarding the scope of BPA's potential effects on these key factors in embryogenesis, but with apparently no focus specifically on the safety assessment of BPA. As such, however, this study did report suggestive findings regarding an inverted U dose response for BPA's effects on m-RNA expression. This information may serve to help identify potential sensitive endpoints for subsequent studies. Efforts to assess possible effects of BPA should utilize well-designed safety assessment animal protocols that will also provide appropriate correlative information to determine biological relevance and appropriate dose-response, including nonmonotonic, information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Patisaul et al (2006)

Scientific Merit:

Positive Features:

1. A positive control (17 β-estradiol) for the BPA treatment was used. An effect dose of E2 able to masculinize female pups (based on number of tyrosine

hydroxylase-immunoreactive cells in the anteroventral periventricular nucleus of the hypothalamus (AVPV)) was validated in the authors' laboratory prior to use in this study.
2. Analyses were carried out on male and female neonates.
3. Animals were fed a soy-free, phytoestrogen-free diet, minimizing exposure of the experimental animals to contaminant sources of estrogenic chemicals.

Negative Features and Issues Impacting Interpretability:

1. BPA was administered to each neonate subcutaneously and only at a single treatment level of 500 µg/pup/day on PND 1 and PND 2 (BPA was actually administered to each pup at a treatment level of 250 µg/pup once every 12 hours for 48 hours). The estimated dose level, assuming a 10 g pup, was approximately 50 mg/kg/day. Without dose response information, interpretation of the treatment effects is difficult and a NOAEL cannot be determined.
2. A total of 5 pregnant rats was allowed to litter. At PND 0, all pups were cross-fostered among the 5 dams. Only four of the dams were given equal sexes of 6 male and 6 female pups: one dam was given only 5 male pups with no females. All pups were then randomly assigned to treatment groups (control, E2 positive control, BPA 500 µg/pup/day, and genistein 500 µg/pup/day) at n=5 – 8 per sex per treatment group. It is not known whether the random assignment of the 5 male pups from the dam/litter without female siblings to treatment groups with male pups from litters with female siblings may have had any confounding effects on the measurements.
3. There was no indication that the investigator doing the immunohistochemical cell counts was blind to treatment conditions, thereby raising the possibility of unintentional bias in collection of data.
4. There were no concomitant neurochemical analyses, endocrine measures or behavioral/functional assessments of the experimental animals with which to correlate the treatment related effects on tyrosine hydroxylase immunoreactive (TH-ir) and estrogen receptor α (ER-α) cells in the anteroventral periventricular nucleus of the hypothalamus (AVPV).
5. There was no information about whether the purity of the drinking water was determined. Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The focus of this study on changes in the development of the sexually dimorphic AVPV of the hypothalamus is relevant to sexual differentiation in the developing mammalian brain, including humans. However, until additional information is developed to clarify the biological significance of the BPA related changes in TH and estrogen receptor patterns in the AVPV of the hypothalamus, it is not possible to interpret or extrapolate the biological relevance or significance of these changes to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations in the experimental design of this study, in particular involving route of administration, dose response information and correlative measures to assess biological relevance, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at 50 mg/kg/day, the lowest dose used in this study, it is questionable whether the designation of LOAEL for this dose should be made, since this study utilized only one treatment level, subcutaneous and not oral dosing was used, and this study was not designed as a safety assessment study.

The nature of this research study appears to be focused on hypothesis testing that exposure to endocrine active chemicals during the first few days after birth (in the rodent), a critical period in mammalian development, could alter the sexually dimorphic expression of TH cells and overall expression of estrogen receptor α in the AVPV of the hypothalamus, but with apparently no specific focus on the safety assessment of BPA. As such, however, this study did report suggestive findings that TH expression patterns in the developing AVPV are sensitive to disruption by endocrine active chemicals and may be a reliable, early marker for examining effects of compounds such as BPA on the neonatal brain. Efforts to utilize such measures in assessing possible developmental effects of BPA should employ well-designed safety assessment animal protocols that will also provide appropriate routes of administration, correlative information to determine biological relevance, and appropriate dose-response information to estimate reliably the applicable LOAEL and NOAEL for BPA.

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Patisaul et al (2007)

Scientific Merit:

Positive Features:

1. All pups were randomly assigned to treatment groups (control, E2 positive control, BPA 500 µg/pup/day, and genistein 500 µg/pup/day) at n=5 – 8 male pups. Also, to prevent cross-contamination, each litter of cross-fostered pups contained only one treatment group.
2. Animals were fed a soy-free, phytoestrogen-free diet, minimizing exposure of the experimental animals to contaminant sources of estrogenic chemicals.

Negative Features and Issues Impacting Interpretability:

1. BPA was administered to each neonate subcutaneously and only at a single treatment level of 500 µg/pup/day on PND 1 and PND 2 (BPA was actually administered to each pup at a treatment level of 250 µg/pup once every 12 hours for 48 hours). The estimated dose level, assuming a 10 g pup, was approximately 50 mg/kg/day (although the authors stated that the dose level

was twice the 50 mg/kg/day level). Without dose response information, interpretation of the treatment effects is difficult and a NOAEL cannot be determined.

2. A total of 5 pregnant rats was allowed to litter. At PND 0, all pups were cross-fostered among the 5 dams. Four of the dams were given 6 male and 6 female pups, and the fifth dam received 2 males and 2 females.
3. The number of male pups used (n=5-8 per group) is a minimum number of animals for reliable data.
4. Experimental treatment and testing in this study was done only with male animals. However, an age-matched group of ovariectomized female offspring (assumingly from the control litters, but this was not specifically stated by the authors) were used as a comparative control group to show the normal response in adult females to estrogen/progesterone activation of GnRH neurons (which are functionally linked to the AVPV).
5. A positive control for the estrogenic effects of neonatal treatment with BPA (e.g., use of estradiol) was not included in this study. In the absence of a concomitant positive control treatment in the neonatal animals, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA when administered to the neonatal animal.
6. There was no indication that the investigator doing the immunohistochemical cell counts was blind to treatment conditions, thereby raising the possibility of unintentional bias in collection of data.
7. There were no concomitant neurochemical analyses, endocrine measures or behavioral assessments of the experimental animals with which to correlate the treatment related effects of neonatal exposure to BPA on the morphology, neuronal function, and cell phenotype within two sexually dimorphic brain regions in adult male rats, namely the anteroventral periventricular nucleus of the hypothalamus (AVPV) and the sexually dimorphic nucleus of the preoptic area (SDN).
8. There was no information about whether the purity of the drinking water was determined. Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The focus of this study on the morphology, neuronal function, and cell phenotype in the development of two sexually dimorphic regions of the brain (AVPV and SDN) is relevant to sexual differentiation in the developing mammalian brain, including humans. However, until a more appropriate experimental design is used (specifically regarding dose levels and route of administration) to replicate the findings from this study and additional information developed to clarify the biological significance of the BPA related changes in neuronal function and cell phenotype in the development of the AVPV and SDN, it is not possible to interpret or extrapolate the biological relevance or significance of these changes to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations in the experimental design of this study, in particular involving route of administration, dose response information and correlative measures to assess biological relevance, this study has little direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at an estimated dose of 50 mg/kg/day, it is questionable whether the designation of LOAEL for this dose should be made, since this study utilized only one treatment level, the route of administration was subcutaneous and not oral, and this study was not designed as a safety assessment study.

The nature of this research study appeared to be focused on hypothesis testing that exposure to BPA during the critical early neonatal period could disrupt brain sexual differentiation and on developing a more sensitive and comprehensive assessment of treatment related changes in the development of sexually dimorphic brain circuits, but with apparently no specific focus on the safety assessment of BPA. As such, however, this study did report suggestive findings that in assessing the developmental disruption of sexually dimorphic nuclei in the brain by endocrine-active compounds, such as BPA, morphologic analyses do not reliably predict changes in neuronal function or disruption of cell phenotype within these nuclei. Efforts to assess possible developmental effects of BPA should therefore employ well-designed safety assessment animal protocols utilizing a comprehensive array of morphologic and functional biomarkers with an appropriate route of administration, correlative information to determine biological relevance, and a dose-response range sufficient to reliably estimate the applicable LOAEL and NOAEL for BPA.

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Porrini et al (2005)

Scientific Merit:

Positive Features:

1. BPA treatment was administered orally with a micropipette.
2. The behavioral observations were scored by an observer blind to experimental treatment.

Negative Features and Issues Impacting Interpretability:

1. BPA was administered to pregnant rats only at a single treatment level of 40 µg/kg/day. Rats were dosed orally from the time of mating until weaning of the pups (PND 21). Without dose response information, interpretation of the treatment effects is difficult and a NOAEL cannot be determined.
2. A total of 22 adult females were randomly allocated to two treatment groups: BPA (n=12) and Oil (n=10) and mated. A total of 18 female offspring in the

BPA group and 18 in the Oil group was used for testing. Apparently more than one animal per litter was used for testing and the individual pup was used as the statistical unit. It is not appropriate to count pups from the same litter as separate subjects in data analyses.

3. Only female offspring were used for data collection.
4. There was no positive control for the estrogenic effects of developmental treatment with BPA. In the absence of a concomitant positive control treatment, it is not possible to gauge the sensitivity of the animal model and species specific endpoints used in this study to the estrogenic types of effects attributed to BPA.
5. There was also no positive control for the test method or behavioral endpoints to gauge the validity and sensitivity of the behavioral observations for detecting treatment related changes.
6. There were no concomitant morphochemical evaluations, endocrine measures or assessments of primary behavioral domains with which to correlate the reported effects of developmental exposure to BPA on the species-specific social/non-social behaviors.
7. There was no information about whether the food fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens) or whether the purity of the drinking water was determined. Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The focus of this study on species specific animal social/non-social behaviors has only indirect relevance to humans insofar as changes in such animal behaviors may reflect some general biological influence of treatment in the animal model. However, in the absence of any clear understanding of validity or general biological significance of changes in species specific animal social/non-social behaviors, it is not possible to interpret or extrapolate the significance of these types of changes to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations in the experimental design of this study, in particular questions regarding the validity of the species specific behavioral observations, the route of administration, the lack of dose response information and correlative measures, this study has no safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at a dose of 40 µg/kg/day, it is questionable whether the designation of LOAEL for this dose should be made, since this study utilized only one treatment level, the route of administration was subcutaneous and not oral, there are uncertainties regarding the validity of the endpoints used, and this study was not designed as a safety assessment study.

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Razzoli et al (2005)

Scientific Merit:

Positive Features:

1. BPA treatment was administered orally to adult female gerbils with a micropipette at two dose levels, 2 and 20 μg/kg/day, plus an oil control, and 17α-ethinyl estradiol (0.04 μg/kg/day; 17αE) was used as a positive control for the estrogenic effects of BPA treatment (n=12 per group).
2. The behavioral observations in the free exploratory test were scored by an observer blind to experimental treatment.

Negative Features and Issues Impacting Interpretability:

1. Only adult female gerbils were used for data collection.
2. There is no indication that the observer scoring the social/non-social behaviors was blind to experimental treatment (although the observer scoring the exploratory test was described as being "experimentally blind").
3. Each treated female was paired throughout the three weeks of daily BPA dosing with an untreated male. The social/non-social behavioral interactions of each male/female pair were determined, but only the female behaviors were analyzed for treatment related effects. The possibility exists that the male was indirectly exposed to BPA at an indeterminate level through co-habitation contact with the dosed female, thereby confounding interpretation of the noted treatment related changes in female social behavior. It is possible that indirect exposure of the male to BPA may have somehow changed the social dynamic between the male and female pair.
4. There was no positive control for the test method or behavioral endpoints to gauge the validity and sensitivity of the species specific social behavioral observations or the exploratory test in the gerbil animal model for detecting treatment related changes.
5. There were no concomitant morphochemical evaluations or endocrine measures with which to correlate the reported effects of BPA on the species-specific social/non-social behaviors and exploratory behaviors of the adult gerbil.
6. There was no information about whether the food fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens) or whether the purity of the drinking water was determined. Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.

Relevance to Humans:

The focus of this study on species specific animal social/non-social behaviors has no specific relevance to humans but changes in these behaviors may reflect some general biological influence of treatment in the animal model. However, in the

absence of any clear understanding of validity or general biological significance of changes in species specific animal social/non-social behaviors, it is not possible to interpret or extrapolate the significance of these types of changes to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations in the experimental design of this study, in particular questions regarding the relevance and validity of the species specific behavioral observations and the lack of correlative measures, this study has no safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the lower dose level of 2 µg/kg/day, it is questionable whether the designation of LOAEL for this dose is appropriate, since there are uncertainties regarding the relevance and validity of the species specific endpoints used, and this study was not designed as a safety assessment study.

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Rubin et al (2006)

Scientific Merit:

Positive Features:

1. There are two unique aspects of this study which are particularly noteworthy: (1) detection of possible treatment effects were based on combined measures of sexually dimorphic histochemical and behavioral endpoints, and (2) specific steps were taken to minimize the unintentional exposure of the experimental animals to estrogenic contaminants from food, caging, bedding material and water bottles.
2. Two dose levels of BPA were used (0.025 and 0.25 µg/kg/day).
3. Treatment related information about numbers of pups and the proportion of male/female at birth were recorded and analyzed. Only litters with a normal distribution of sexes at birth were included in this study.
4. Efforts were made to use pairs of male and female offspring from different litters for the histochemical analyses and the behavioral testing at 6-9 weeks of age to eliminate the confounding influence of litter effect.
5. The histochemical analyses were conducted by several observers blind to the sex and treatment of the animals.

Negative Features and Issues Impacting Interpretability:

1. BPA was administered to pregnant mice subcutaneously via an implanted osmotic pump designed to deliver 0.025 or 0.25 mg BPA/kg/day from Gestation Day 8 until Postnatal Day 16. BPA was dissolved in 50% DMSO and the vehicle control group of animals received 50% DMSO.
2. A saline group was not included as a control for any possible effects of the 50% DMSO vehicle. In the absence of a saline control, it is not known

whether the DMSO vehicle may have had some effect on the dependent measures used in this study.

3. There was no positive control for the estrogenic effects of developmental treatment with BPA. In the absence of a concomitant positive control treatment, it is not possible to gauge the sensitivity of the animal model and endpoints used in this laboratory to the estrogenic types of effects attributed to BPA.
4. There was no indication that the observer(s) scoring the open-field behaviors were blind to the sex or experimental treatment of the test animals.
5. One of the primary behavior endpoints in open-field testing is a measure of horizontal movement (typically, number of squares crossed or number of peripheral photobeams crossed). However, this endpoint was not used in the present study apparently because scoring of the open-field behaviors was done manually by observers and not with an automated monitoring system. Movement of the animals was too rapid for the observers to make accurate measures of squares crossed.
6. There were no concomitant endocrine measures or assessment of sexual development which would have provided additional supportive information with which to correlate the reported effects of BPA on TH neurons in the AVPV and the sexually dimorphic behaviors in the open-field test.

Relevance to Humans:

The focus of this study on the ability of chronic low-level prenatal exposure to BPA to affect the development of sexually dimorphic brain regions and correlative behavioral measures could be of relevance to humans, pending verification and replication of the findings of this study using a more appropriate experimental design and clarification of the scope of effects.

Utility for Food Additive Regulatory Decisions:

Due to the limitations in the experimental design of this study, in particular regarding the route of administration and the absence of a critical control, this study information has no direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the lower dose level of 0.025 µg/kg/day, it is questionable whether the designation of LOAEL for this dose is appropriate, particularly since the route of administration was subcutaneous (implanted osmotic pump) and this study was not designed as a safety assessment study.

The nature of this research study appeared to be focused on exploring the ability of prolonged low level perinatal exposure to BPA to affect anatomical and functional measures of brain development and sexual differentiation, but with apparently no specific focus on the safety assessment of BPA. As such, however, this study did report suggestive findings that BPA may affect the development of a sexually dimorphic region of the brain (specifically involving tyrosine hydroxylase neurons) and sexually dimorphic behaviors in the juvenile and adult female offspring. Efforts should be made to replicate and extend these findings

with well-designed safety assessment animal protocols utilizing sexually dimorphic morphologic (histochemical) and functional behavioral biomarkers with an appropriate route of administration, correlative endocrine and information about sexual development, and a dose-response range sufficient to estimate the applicable LOAEL and NOAEL for BPA.

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Ryan BC (2005 – Thesis Dissertation), Ryan and Vandenbergh (2006) and Ryan et al (2006 - Abstract)

(Note that the basic report being critiqued [*Ryan, 2005 which was a thesis dissertation*] consisted of two separate studies, one using mice and the other rats. Consequently, this critique will have two component parts, the first dealing with the mouse study and the second with the rat study. Since *Ryan and Vandenbergh, 2006* was a full publication presenting the same mouse data from the *Ryan (2005)* dissertation, the section of the mouse study critique below applies to both *Ryan (2005)* and *Ryan and Vandenbergh (2006)*. *Ryan et al, 2006* was a scientific meeting abstract of the rat study results from *Ryan (2005)* and, consequently, comments relevant to this abstracted information are contained in the rat study critique below. It should also be noted that, unfortunately, the dissimilarities in the procedures and test methods used between the mouse and rat studies make it very difficult to compare the relative sensitivities of these two animal models to the developmental effects of estrogenic chemicals, such as estradiol and BPA.)

MOUSE STUDY (Ryan, 2005; Ryan and Vandenbergh, 2006)

Scientific Merit (mouse study):

Positive Features:

1. Two dose levels of BPA were used (2 and 200 µg/kg/day) administered orally (gavage) to pregnant mice daily from Gestation Day 3 until Postnatal Day 21.
2. A positive control (5 µg ethinyl estradiol/kg/day; EE) for the estrogenic effects of developmental treatment with BPA was included.
3. Pup body weight and litter size were included in the study as endpoints of general toxicity.
4. The assessment of endocrine disrupting effects from developmental exposure to BPA included reproductive endpoints (time to onset of female puberty based on first estrus cycle and anogenital distance) and sexually dimorphic behaviors, specifically anxiety (assessed in two tests, the elevated plus maze and light/dark preference) and spatial memory (assessed in two tests, the radial arm maze and the Barnes maze).
5. Behavioral testing was conducted using ovariectomized female mice.

Negative Features and Issues Impacting Interpretability:

1. The number of pregnant dams dosed per group was not specified. Also, the numbers of litters per group was also not specifically stated in the Materials and Methods, but in the presentation of general reproduction data in Table 1 (publication) it appears that there were 16 litters per treatment group.

2. Litters were apparently not culled. Consequently, there was no standardization of the number of pups or the proportion of male and female pups per litter. It is possible that variable sizes of litters (e.g., nutritional and socialization factors) and variable ratios of male/female pups (socio-sexual factors) in litters could have an effect on animal performance in the behavioral tests.
3. A number of the treatment related findings seemed to be based on unplanned post-hoc statistical analyses rather than planned analyses.
4. The investigator acknowledged the potential confounding influence of possible contaminant sources of estrogenic chemicals, including standard rodent chow (phytoestrogens) and polycarbonate cages and plastic water bottles (BPA). Yet, all of these items were used in the housing of the experimental animals and there were no apparent attempts to conduct blood/tissue or environmental analyses to determine the occurrence and/or extent of estrogenic chemical contamination in the experimental animals. This certainly introduces the possibility of a confound in interpretation of the study findings.
5. While pup weight, litter size and anogenital distance (AGD) were used as endpoints of general/reproductive toxicity, all of these measures were made in this study at the time of weaning. These data do not provide any information about pup mortality or transient changes in pup body weight that could have occurred during the first three weeks of neonatal life. Traditionally, these measures are made at or soon after birth to provide more meaningful information.
6. Based on data presented, the litter sizes for all treatment groups measured at the time of weaning averaged approximately 7-8 pups per litter (sex proportion was not presented). This appears to be an unusually small average litter size.
7. The determination of onset of puberty (based on age of first estrus cycle) used very small numbers of animals, with n's of 5, 4, 5 and 7 in the control, BPA 2, BPA 200 and EE treatment groups, respectively. With such small numbers of subjects, confidence in the reliability of findings is low.
8. The authors mentioned that the mice in this study inexplicably reached puberty precociously (compared with other published data). Although not mentioned by the authors, it could be speculated that this precocious puberty in females could be related to the possible exposure to estrogenic contaminants through the food, caging and water bottles.
9. The same female offspring were used for both tests of "anxiety" and another set of animals was used for both tests of spatial memory, raising questions of 'test-test' interactions and differences between treated and control mice.
10. The author's discussion of the light/dark preference test appears to interpret the treatment related decreased time spent in the lighted chamber as a direct measure of "anxiety". "Anxiety" is more appropriately used to refer to a psychological state in humans. The amount of time an animal spends in a light or dark chamber is simply a quantification of preferential behavior in animals. While a significant change in such preferential behavior may be viewed as a functional index of some alterations in the processing of certain brain

circuitry, it should not necessarily be interpreted as a specific index of anxiety-like (fear associated) behavior since many other behavioral factors (e.g., activity, cognitive function, level of motivation, distractibility, sensory cues, etc.) may influence the amount of time an animal spends in a light or dark environment. Although, time in the lighted area of this type apparatus is sensitive to anxiolytic and anxiogenic compounds which provides a certain amount of evidence that this behavior is at least anxiety-related.

11. There was no indication that the observer(s) scoring the behaviors were blind to the experimental treatment of the test animals.
12. There were no concomitant endocrine or morphochemical measures with which to correlate the reported reproductive and behavioral effects of BPA.

Relevance to Humans:

The focus of this study on the ability of chronic low-level developmental exposure to estrogenic chemicals, such as ethinyl estradiol and BPA, to affect the development of reproductive endpoints and sexually dimorphic behavior is relevant to sexual differentiation in the developing mammalian brain, including humans. However, until a more appropriate experimental design is used to replicate the more salient findings from this study and clarify their biological relevance, it is not possible to interpret or extrapolate the significance of these findings to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations in the experimental design and exploratory nature of this study, the findings have little direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although limited treatment related findings were reported in this study at a dose of 200 µg/kg/day, it is questionable whether the designation of LOAEL for this dose should be made, since the biological relevance of the findings associated with this dose (slight early onset of puberty and preference for dark chamber) are unclear, and this study was not designed as a safety assessment study.

The nature of this research study focused on exploring the utility of behavioral measures in assessing the endocrine disrupting effects of developmental exposure to environmental estrogens, such as BPA and ethinyl estradiol, and identifying sensitive procedures, but with apparently no specific focus on the safety assessment of BPA. As such, however, this study did report suggestive findings that certain reproductive endpoints and sexually dimorphic behaviors may be sensitive to the developmental effects of BPA. Efforts to assess the developmental neurotoxicity of BPA could consider the utility of similar endpoints and procedures in well-designed safety assessment animal protocols.

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***RAT STUDY* (Ryan, 2005)**

Scientific Merit (rat study):

Positive Features:

1. Several dose levels of BPA were used (2, 20 and 200 µg/kg/day) administered orally (gavage) to pregnant rats daily from Gestation Day 7 until Postnatal Day 18.
2. Ethinyl estradiol (EE) was used as a positive control for the estrogenic effects of developmental treatment with BPA. EE was administered orally (gavage) at several dose levels: 0.05, 0.5, 5 and 50 µg/kg/day to pregnant rats daily from GD 7 until PND 18.
3. Litter size, sex ratio, number of uterine implant sites, pup mortality, pup body weight, and number of areola (nipples), each measured at relatively appropriate ages, were included in the study as endpoints of general toxicity.
4. The assessment of endocrine disrupting effects from developmental exposure to BPA included reproductive endpoints (anogenital distance (AGD) and onset of puberty in females based on age of vaginal opening) and sexually dimorphic behaviors, specifically motor activity and saccharine preference.
5. Behavioral testing was conducted using ovariectomized female rats.

Negative Features and Issues Impacting Interpretability:

1. Most, if not all, of the measures of general reproductive toxicity and the behavioral tests each appeared to use multiple animals (females) from the same litter. The basic planned statistical analyses (although not completely described) seemed to use the individual pup/offspring as the statistical unit and not the litter. Several post-hoc statistical analyses were added that used litter means.
2. There was no description of culling litters to standardize litter size to a specified number of pups with an equivalent proportion of male and female pups per litter.
3. In the comparison of estrogenicity of several oil vehicles using the immature rat uterotrophic assay, an EE control group was not included (in contrast to the mouse study in which an EE control was used which provided some information with which to gauge the basic sensitivity of the animal model to estrogenic chemicals).
4. A number of the treatment related findings seemed to be based on unplanned post-hoc statistical analyses rather than planned analyses.
5. Behavioral testing was basically restricted to female offspring of treated mothers.
6. None of the results of AGD measurements or areola counts were reported or discussed for BPA.
7. No tests of cognitive function were carried out in the rat study.
8. Lordosis appeared to be a particularly sensitive measure of EE's developmental reproductive effects. Unfortunately, this behavioral endpoint was not used to assess the developmental effects of BPA.
9. The investigator acknowledged the potential confounding influence of possible contaminant sources of estrogenic chemicals, including standard rodent chow (phytoestrogens) and polycarbonate cages and plastic water bottles (BPA). Yet, all of these items were used in the housing of the

experimental animals and there were no apparent attempts to conduct blood/tissue or environmental analyses to determine the extent of estrogenic chemical contamination in the experimental animals. This certainly introduces the possibility of a confound in interpretation of the study findings.

10. There was no indication that the observer(s) scoring the behaviors were blind to the experimental treatment of the test animals.
11. There were no concomitant endocrine or morphochemical measures with which to correlate the reported effects of BPA.

Relevance to Humans:

The focus of this study on the ability of chronic low-level developmental exposure to estrogenic chemicals, such as ethinyl estradiol and BPA, to affect the development of reproductive endpoints and sexually dimorphic behavior is relevant to sexual differentiation in the developing mammalian brain, including humans. However, until a more appropriate experimental design is used to replicate the more salient findings from this study and clarify their biological relevance, it is not possible to interpret or extrapolate the significance of these findings to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations in the experimental design and exploratory nature of this study and the fact that, in contrast to ethinyl estradiol, BPA gave inconsistent results and showed no clear dose response throughout the (rat) study, the findings have little direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at a dose of 2 µg/kg/day, it is questionable whether the designation of LOAEL for this dose should be made, since the high variability of the motor activity data and the biological relevance of the singular non-dose related finding associated with BPA (decreased response to estradiol stimulation of motor activity in adult female offspring) are unclear, and this study was not designed as a safety assessment study. Due to the exploratory nature of this study it is also questionable whether the designation of a NOAEL is appropriate.

The nature of this research study focused on exploring the utility of behavioral measures in assessing the endocrine disrupting effects of developmental exposure to environmental estrogens, such as BPA and ethinyl estradiol, and identifying sensitive procedures, but with apparently no specific focus on the safety assessment of BPA. As such, however, this study did report suggestive findings that certain reproductive endpoints and sexually dimorphic behaviors may be sensitive to the developmental effects of environmental estrogens, particularly ethinyl estradiol and possibly BPA. Efforts to assess the developmental neurotoxicity of BPA could consider the utility of similar endpoints and procedures in well-designed safety assessment animal protocols.

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Shikimi et al (2004)

Scientific Merit:

Positive Features:

1. Two dose levels of BPA were used (50 and 500 µg/day).
2. A positive control (5 µg estradiol benzoate/day; EB) for the estrogenic effects of BPA was included.
3. To analyze mode of action of the effects of BPA, tamoxifen (TXF), an estrogen receptor antagonist, was included.
4. Both male and female pups were used.

Negative Features and Issues Impacting Interpretability:

1. All treatments with BPA, EB and TXF were injected into the cerebrospinal fluid around the posterior vermal lobe (IX) of the cerebellum of the pups daily from PND 6 to 9.
2. The procedure for injecting the test substances into the cerebrospinal fluid to ensure accurate and replicable placement of the needle for each injection and for each animal was not described. There was also no discussion of a marker injected to ensure accurate placement of the needle.
3. All treatment groups consisted of n=4 pups per sex, a rather low number of subjects.
4. The number of litters used for this study was not specified. Also, it was not stated whether the pups per group were taken from different litters or the same litter, or whether the treated pups were kept in separate or common litters. These specifics are important since the authors stated that the statistical analyses were based on the individual animal, not the litter.
5. There was no specific information regarding whether litters were culled to standardize litters to a certain size or with an equivalent proportion of male and female pups per litter.
6. There was no information about housing or whether the food fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens) or whether the purity of the drinking water was determined. Also, regarding another potential source of contaminant exposure, the types of caging and water bottles used during the study were not specified. Bisphenol A is a known component in polycarbonate materials.
7. There was no indication that the investigator conducting the histological analyses was blind to the sex and experimental treatment of the test animals.
8. There were no concomitant functional measures with which to correlate and assess biological significance of the stimulatory effects of BPA on Purkinje cell dendritic outgrowth.

Relevance to Humans:

The focus of this study on the effects of xenoestrogens on the growth of Purkinje cells *in vivo* using newborn rats is relevant to the basic biology of steroidal

involvement in cerebellar development. The relevance of this study lies in the potential of this model for studying the actions of xenoestrogens.

Utility for Food Additive Regulatory Decisions:

The findings of this study using direct injections into the cerebrospinal fluid of neonatal rats have no direct safety assessment utility for supporting a regulatory decision on BPA.

The nature of this research study focused on exploring the in vivo effects of xenoestrogens (including BPA) on the growth of cerebellar Purkinje cells using newborn rats, but was not intended to assess safety of BPA. As such, however, this study did report findings that in vivo treatment with BPA in newborn rats promote dendritic outgrowth of cerebellar Purkinje cells through an estrogenic mode of action. Since Purkinje cells play a role in the process of learning and memory, this study's results suggest that future studies of xenoestrogens focus attention on behavior, as well as histomorphology.

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Tando et al (2007)

Scientific Merit:

Positive Features:

1. Two dietary levels of BPA were used in this study (i.e., 0.003 and 8 mg BPA/g food (= 3 and 8000 mg/kg food)). Experimental diets were given to pregnant mice from day of conception until weaning (PND 21).
2. Male and female offspring were evaluated in the study.

Negative Features and Issues Impacting Interpretability:

1. Actual food intake and calculation of daily dose levels were not reported by the study authors, but estimated dose levels were 0, 0.6 and 1600 mg/kg body weight/day for the dietary levels of 0 (control), 0.003 and 8 mg BPA/g food, respectively, based on the assumption that a female mouse consumes approximately 0.2 kg food/kg body weight/day.
2. Effects on the offspring were confounded by potential direct exposure of the pups to the treated feed as they started eating on their own late in lactation.
3. There was no positive control for the estrogenic effects of developmental treatment with BPA. In the absence of a concomitant positive control treatment, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic types of effects attributed to BPA.
4. Treatment groups consisted of 4 to 5 pups per sex per group, a rather low number of subjects.
5. The number of litters used for this study was not specified. Also, it was not stated whether the pups per group were taken from different litters or the same

litter. These specifics are important since it appears that the statistical analyses were based on the individual animal, not the litter.

6. There was no specific information regarding whether litters were culled to standardize litters to a certain size or with an equivalent proportion of male and female pups per litter.
7. After weaning, all pups were housed up to 8-11 weeks of age, assumingly (although not specifically stated) when they were sacrificed for immunohistochemical analyses. However, it is not stated whether animals were housed individually or in groups, or whether same sex littermates were housed together.
8. There was no indication that the investigator conducting the immuno-histological analyses was blind to either the sex or treatment of the subject animals.
9. The specific reason given by the authors for choosing analysis of dopaminergic neurons in the substantia nigra for study is that the substantia nigra is one of the sexually dimorphic nuclei in rodent brain. Yet, the immunohistochemical results for the control animals showed no significant sex related differences. The implications of the control animals not showing evidence of sexually dimorphism in the substantia nigra to the interpretation of the decreased TH-ir neurons in this brain region for the 0.003 mg BPA/g diet level group was not discussed by the authors.
10. There was no information presented about the type of caging and water bottles (e.g., polycarbonate, polypropylene, etc.), or whether the food fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). These represent potential sources of contaminant exposure, particularly the types of caging and water bottles used since BPA is a known component of polycarbonate materials.
11. There was no indication that the investigator conducting the histological analyses was blind to the sex and experimental treatment of the test animals.
12. There were no concomitant neurochemical, endocrine or functional measures with which to correlate and assess the biological significance of the reported decrease in TH-ir neurons in the substantia nigra of only females at the low but not higher dietary level of BPA.

Relevance to Humans:

The endpoints in this study, involving development of the sexually dimorphic brain dopaminergic system in the substantia nigra, are relevant to mammalian nervous system function, including humans. However, until clarification can be provided regarding the procedures and methods used in this study and the biological significance of the selective effect in females and only at the lower treatment level of BPA, it is not possible to interpret the relevance of the treatment related changes reported or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, particularly in its experimental design and the absence of critical information regarding study procedures and methods, this

study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at dietary dose levels as low as approximately 0.6 mg/kg/day (based on dietary concentration of 0.3 mg BPA/kg food), it is questionable whether the designation of LOAEL for this dose should be made with any confidence, since critical information regarding the procedures and methods in the study were not provided and since this study was not specifically designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding BPA's potential developmental effects on sexually dimorphic nuclei in the brain, and less on specific safety assessment. As such, however, this study did report suggestive findings associated with effects of prenatal/postnatal dietary exposure to BPA, specifically the decrease in substantial nigra dopaminergic neurons in female (not male) mice at low but not higher dose levels of BPA. This information may serve to help identify potential sensitive endpoints for subsequent studies to assess the developmental neurotoxic effects of BPA. Such studies should incorporate sensitive endpoints in well-designed safety assessment animal protocols that will also provide appropriate dose-response information to delineate any nonmonotonic dose-response effects and estimate the applicable LOAEL and NOAEL for BPA.

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Xu et al (2007)

Scientific Merit:

Positive Features:

1. Two levels of BPA in drinking water were used in this study (i.e., 0.1 and 50 mg BPA/L water). BPA drinking water was given to pregnant rats from GD 11 until weaning (PND 21).
2. Male and female offspring were evaluated in the study.
3. A profile of treatment related effects was determined, based on hormonal analyses, PCR assessment of mRNA expression, immunocytochemical evaluation, determination of protein expression, and behavioral testing.

Negative Features and Issues Impacting Interpretability:

1. Although it was stated that there were no treatment effects on water consumption, actual water intake data were not presented and calculation of daily dose levels were not reported by the study investigators, but estimated dose levels were 0, 0.02 and 10 mg/kg/day for the drinking water levels of 0 (control), 0.1 and 50 mg BPA/L water, respectively, based on a rat average water consumption of 0.05 L/day and assuming body weight of 0.25 kg.

2. Effects on the offspring were confounded by potential direct exposure of the pups to the treated drinking water as they started drinking on their own late in lactation.
3. There was no positive control for the estrogenic effects of developmental treatment with BPA. In the absence of a concomitant positive control treatment, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic effects attributed to BPA.
4. In making the BPA drinking water solutions, BPA was dissolved in 0.01% ethanol in un-chlorinated pure water. However, the only control group used in this study was given tap water. The latter is an inadequate control for the vehicle used in the BPA drinking water and at best confounds the interpretation of the data. Specifically, it is unknown whether the 0.01% ethanol itself, rather than the BPA, could have affected any or all of the endpoints being measured in this study.
5. While the number of litters used for this study was specified (n=8 – 9 dams/group), it was not stated whether the pups used for each of the various tests were taken from different litters (i.e., maintaining litter as the statistical unit) or the same litter (i.e., individual pup used as statistical unit). However, for the open-field testing, an n=13-17 per group and for T4 analyses the n=10-14 per group, indicating that multiple pups for each of these tests were taken from the same litter. Using individual pups as the statistical unit in a developmental study artificially inflates the power of the statistical test.
6. There was no specific information regarding whether litters were culled to standardize litters to a certain size or with an equivalent proportion of male and female pups per litter.
7. There was no indication that the investigators recording behaviors in the open-field and Morris water maze tests, or conducting the immuno-histological analyses were blind to either the sex or treatment of the subject animals, raising the possibility of unintentional bias in recording the treatment related effects.
8. There was no information about whether water bottles were plastic (i.e., polycarbonate or other material), or whether the food fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). These represent potential sources of contaminant exposure to xenoestrogens.
9. The analysis of expression of mRNA for THR, RC3/neurogranin and SRC-1 was carried out only for males and only for the 0.1 mg BPA/L treatment group. This same type of information for the 50 mg BPA/L group would have enabled a more reliable assessment and evaluation of the apparent nonmonotonic dose response effects of BPA treatment.

Relevance to Humans:

The endpoints in this study, involving developmental changes in behavior and thyroid hormone pathways, are relevant to the functional development of the mammalian nervous system, including humans. However, until confirmation of these effects can be made using appropriate controls and clarification provided for the biological significance of the reported nonmonotonic effects of BPA, it is not

possible to interpret the relevance of the treatment related changes reported or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study's experimental design, particularly involving an inappropriate control group, this study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at drinking water dose levels as low as approximately 0.02 mg/kg/day (based on a drinking water level of 0.1 mg BPA/L water), it is questionable whether the designation of LOAEL for this dose should be made with any confidence, since the study control was inadequate and this study was not specifically designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding BPA's potential neurodevelopmental effects as related to thyroid function, and less on specific safety assessment. As such, however, this study did report suggestive findings associated with effects of prenatal/postnatal dietary exposure to BPA, specifically regarding the sex-specific nonmonotonic dose-related treatment effects for BPA. This information may serve to help formulate future studies of the neurodevelopmental effects of BPA. Such studies should use well-designed safety assessment animal protocols that will also provide appropriate dose-response information to delineate any nonmonotonic dose-response effects and estimate the applicable LOAEL and NOAEL for BPA.

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Zoeller et al (2005)

Scientific Merit:

Positive Features:

1. Three dose levels of BPA administered orally in food were used (i.e., 0, 1, 10 and 50 mg BPA/kg). An appropriate solution of BPA in methanol was pipetted onto a food wafer (which was allowed to dry in a fume hood) and fed to pregnant rats daily from GD 6 until weaning (PND 21).
2. Male and female offspring were used for the serum T4 analyses but, since there was no gender effect in the statistical analyses, only males were evaluated for serum TSH levels and RC3/neurogranin mRNA expression.
3. Dam body weights during gestation and lactation and pup (male and female) body weights during lactation were monitored.

Negative Features and Issues Impacting Interpretability:

1. BPA doses were administered by feeding each of the rat dams a food wafer containing the appropriate BPA dose. However, there was no mention of verification that the animals consumed the wafers. Also, there was no

presentation regarding general food intake (this would have helped interpret the dose-related decrease in dam body weight during gestation.)

2. There was no positive control for the estrogenic effects of developmental treatment with BPA. In the absence of a concomitant positive control treatment, it is not possible to gauge the sensitivity of the animal model and endpoints used in this study to the estrogenic effects attributed to BPA. The use of a positive estrogenic control treatment could have provided concomitant experimental data regarding the authors' contention that BPA can selectively affect the thyroid system independent of estrogenic effects.
3. There was no specific information regarding whether litters were culled to standardize litters to a certain size or to an equivalent proportion of male and female pups per litter.
4. There was no information presented about whether the metal caging or water bottles made of polycarbonate or other material, or whether the food fed to the animals was certified to minimize any contaminant estrogenic chemicals (phytoestrogens). These represent potential sources of contaminant exposure to xenoestrogens.

Relevance to Humans:

The endpoints in this study, involving changes in thyroid hormone function during development, are relevant to the functional development of the mammalian nervous system, including humans. However, until clarification can be provided for the biological significance of the reported effects of BPA, it is not possible to interpret the relevance of the treatment related changes reported or to extrapolate their significance to humans.

Utility for Food Additive Regulatory Decisions:

Although there are some limitations of this study's experimental design, upon confirmation the study findings could be considered in a database of information used for the safety assessment of BPA in support of a food additive regulatory decision on BPA. In addition, although treatment related findings were reported in this study at the lowest orally administered dose level used of 1 mg/kg/day, it is questionable whether the designation of LOAEL for this dose should necessarily be used until this study's findings can be replicated and since this particular research was not specifically designed as a safety assessment study.

The nature of this research study appears to be focused more on hypothesis testing regarding the mechanism of action for BPA's potential developmental effects related to thyroid function, and less on specific safety assessment. As such, however, this study did report suggestive findings associated with effects of prenatal/postnatal dietary exposure to BPA, specifically regarding the dose-related increases in serum T4 levels (male and female offspring) and RC3/neurogranin mRNA expression (only male offspring tested). This information may serve to help formulate future studies of the neurodevelopmental

effects of BPA. Such studies should use well-designed safety assessment animal protocols that will also provide appropriate dose-response information to delineate dose-response effects and estimate the applicable LOAEL and NOAEL for BPA.

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Literature Review of Neurobehavioral Effects of Bisphenol A

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Prepared for

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Introduction

The purpose of this report is to a) characterize what is known about the effects of bisphenol A (BPA) on neurobehavioral endpoints based on the papers identified in a literature search conducted by the American Plastics Council (APC); and b) provide an opinion on the weight of evidence concerning effects of BPA on neurobehavioral endpoints at low doses.

The literature on the neurobehavioral effects of BPA is focused almost exclusively on the effects of developmental exposures. This emphasis is due to the fact that BPA has been shown to be weakly estrogenic, with a significantly lower potency than estradiol (EU Summary Risk Assessment Report 2003; Gray et al. 2004). Estrogen plays a role in the appropriate development of the brain and subsequent behavior. A predominant hypothesis regarding estrogenic effects on brain development is that the original phenotype of the brain is female type in mammals, and some areas of the brain are masculinized in male offspring when testosterone is converted to estradiol locally in the brain during a critical perinatal period (Kubo et al. 2003; Schantz et al. 2001). It is hypothesized that the developing female brain is not modified by estradiol during the same period, because circulating estradiol from the dam is bound to alpha-fetoprotein (Kubo et al. 2003). In rodents, this critical period of sexual differentiation of the brain is believed to be a few days before birth to approximately 10 days after birth (Schantz et al. 2001). During this period, the rodent brain is sensitive not only to estrogens but also to direct effects of androgens on the brain. As a result of these hormonal influences, there are normal sex differences in brain structure and function. Sex differences in size and/or structure have been observed in several brain areas, including the sexually dimorphic nucleus of the preoptic area (SDN-POA), ventral medial nucleus of the hypothalamus, and hippocampus. The hypotheses regarding the exact mechanism of sexual differentiation are controversial, and the link between rodents and humans is an area of active research.

Overview of the BPA Neurobehavioral Literature

Eighteen studies that investigated BPA's potential for neurobehavioral effects following developmental exposures were identified and reviewed. The majority of these studies included doses below the U.S. Environmental Protection Agency's (EPA's) lowest-observed-adverse-effect level (LOAEL) of 50 mg/kg-day, mostly in the range between 1 and 10^{-2} mg/kg-day. Fifteen of the studies exposed animals to oral doses (gavage, micropipette, diet, water), which is the most relevant route of exposure to humans. The remaining three non-oral studies injected BPA into dams subcutaneously (Sato et al. 2001), into pups intracisternally (Ishido et al. 2004), and into chicks intracerebrally (Sashihara et al. 2001). Of the 15 oral studies, 11 were conducted on rats and 4 were conducted on mice.

Fifteen studies exposed dams to BPA (14 oral and 1 subcutaneous injection) during gestation and/or lactation. In these studies, the pups were exposed indirectly through the placenta and/or milk. The litter, and not the individual pup, should be the primary experimental unit when evaluating developmental toxicity associated with a maternally administered substance in rodents. Ema et al. (2001) used the largest number of dams (rats, 25/dose group) and evaluated 1 pup/sex/litter from both F1 and F2 pups for ontogeny of sensory/motor behaviors in a two-generation study where the offspring were exposed both indirectly at the fetal and neonatal stages and directly after weaning. The F1 pups were observed in an open field, and six of these pups/sex/group were selected for testing on the Biel water maze. Palanza et al. (2002) also used a sufficiently large number of dams (15–20/dose group), evaluated all the pups from 8 litters/dose group for development (ontogeny) of sensory/motor behaviors and 1 pup/sex/litter for the behavioral tests, and preserved the litter as the statistical unit of analysis even when all pups/litter were evaluated. Negishi et al. (2003, 2004) dosed approximately 8–10 dams and selected 1 pup/litter for behavioral testing. For many of the other studies in which the dams were dosed, the experimental unit was the individual pup and not the litter. The sample size could be as high as 20–30 pups/dose group, but the actual number of litters represented was much lower (e.g., 6–10 dams treated per dose group). In many cases (e.g., Kubo et al. 2003; Suzuki et al. 2003), it was not possible to determine how pups were selected from the different litters to form the treatment groups used for behavioral testing. In general, if the behavioral

measures are of similar quality, studies using sufficiently large numbers of litters/dose group and selecting 1 pup/sex/litter for behavioral testing are more reliable than those using large numbers of pups/dose group from few or an unspecified number of litters/dose group.

One major challenge in evaluating the BPA behavioral data is that, unlike more objective measures such as organ weight, anogenital distance, preputial separation date, etc., very different approaches were used to measure effects on what were reported as the same behavioral endpoint. As a simplified example, "aggression" was measured by one investigator as the total contact time between rats regardless of whether "attacking behavior" was noted. In contrast, other investigators considered some of the behaviors observed during this total contact time as "play" or "attacking." This difference in criteria used to measure aggression can lead to very different conclusions on the effects of BPA on aggression. An additional challenge was that some investigators anthropomorphized the observed behavior (i.e., described animal behavior in terms of human emotions such as anxiety, impulsiveness, motivation), without providing adequate experimental data to support their conclusions. These different approaches make evaluating the corroboration of effects across studies much more complex, because the author's conclusions can be based on unsubstantiated assumptions regarding the specific endpoints measured.

For the purpose of evaluating overall patterns of effects across studies, similar behavioral endpoints as reported by the investigator were grouped together. However, the reader is cautioned to consider the caveats described above and to carefully review Table 1 and Appendix 1 for details on the actual measures evaluated and the strength of the evidence supporting the authors' conclusions regarding effects on the behavioral endpoints.

In order to compare effects, doses were collapsed into general order of magnitude (e.g., 10^{-4}, 10^{-3} mg/kg-day). The actual experimental doses are tabulated in Table 1. Doses were not rounded up to the next higher order of magnitude, because almost all experimental doses used were between 1 and 5 units. The one exception is that the estimated dose level of 75 mg/kg-day based on dietary exposures reported by Suzuki et al. (2003) and Mizuo et al. (2004) was expressed as 10^{1} mg/kg-day rather than rounded up to 10^{2} mg/kg-day.

Studies were not included in the overall weight of evidence if the offspring were dosed intracisternally or intracerebrally. Limited evaluation of sexual behaviors or reproductive or developmental endpoints that may have been included in these studies is provided. Neuropharmacologic and morphometric measures are included, because the behavioral studies that included these endpoints merit attention. However, these non-behavioral endpoints should be evaluated within the context of the larger literature relevant to these endpoints, which task was outside the scope of this review.

The general groups of endpoints identified based on evaluation of the different studies include the following:

- Ontogeny of sensory/motor behaviors and reflexes—grasp reflex, pivoting response, surface righting reflex, mid-air righting reflex, negative geotaxis reflex, straight-line walking, cliff avoidance behavior
- Frequently measured gross behaviors—ambulation, rearing, grooming, defecation
- Complex/cognitive behaviors—Morris water maze, T-maze, active avoidance, passive avoidance, operant behavior
- Complex/emotional behaviors—impulsivity, anxiety, motivation, behaviors in elevated plus maze
- Social behaviors—play, aggression, maternal behavior, sexual behavior
- Pharmacologic challenge—methamphetamine placement preference, morphine placement preference, amphetamine increase in activity, methamphetamine increase in activity, monoamine disruption test on locomotion behavior
- Brain weight and morphometry—diameter of mamillothalamic tract, locus coeruleus, sexually dimorphic nucleus of the preoptic area of the hypothalamus (SDN-POA).

Overall, there were no consistent treatment-related effects in any of the behavioral endpoints that did not involve measuring potentiation or attenuation of behaviors following pharmacologic challenge. Two preliminary findings were of interest but require additional data and replication before any conclusions can be made: 1) BPA potentiated behavioral effects following pharmacologic challenge to methamphetamine at 10^1 and 10^2 mg/kg-day (there were conflicting results at lower doses), and 2) BPA appeared to decrease the sexual difference in the size of the locus coeruleus but not the sexually dimorphic nucleus at 10^{-1} and 10^{-2} mg/kg-day.

The next section discusses the effects of BPA on each of the above general categories of neurotoxicologic endpoints.

Effect of BPA on General Behavioral Endpoints

Table 1 and Appendix 1 provide a more detailed listing of the results. As discussed in more detail in the previous section, dose levels are expressed in terms of order of magnitude, rather than the precise dose levels reported by the investigators. Actual experimental dose levels are provided in Table 1.

Ontogeny of Sensory/Motor Behaviors and Reflexes

Investigators evaluating the impact of BPA exposure on ontogeny of sensory/motor behaviors and reflexes were Ema et al. (2001) and Palanza et al. (2002). Both authors concluded that there were no treatment-related adverse effects of BPA on development of several behaviors. The behaviors and reflexes measured included development of grasp reflex and pivoting response, surface righting reflex, mid-air righting reflex, negative geotaxis reflex, straight-line walking, and cliff avoidance behavior.

Commonly Measured Gross Behaviors

Activity, rearing, grooming, and defecation were commonly measured gross behaviors. These behaviors were often analyzed as separate individual measures. Therefore, we report these effects separately below. However, activity (when defined as horizontal movement), rearing, and grooming are related measurements that need to be evaluated together in terms of a pattern of overall effect. Small decreases in horizontal movement accompanied by small increases in rearing, and vice versa, should not be considered as adverse effects.

Activity

Activity measurements were evaluated by several investigators, including Adriani et al. (2003) (open field activity prior to amphetamine challenge, Figure 4), Aloisi et al. (2002) (following formalin or sham injection into the rat paw), Ema et al. (2001), Farabollini et al. (1999)

(holeboard activity, which is different but related to open field activity), Kubo et al. (2001, 2003), and Negishi et al. (2003, 2004) (12-hour dark phase automated motor activity). Farabollini et al. (1999) noted a statistically significant decrease in the number of squares crossed on the 5-minute holeboard at 10^{-2} mg/kg-day in females, but no effects were noted by Kubo et al. (2001, 2003) in males and females in a 10-minute open field test, by Ema et al. (2001) in males and females in a 3-minute open field study, or by Aloisi et al. (2002) in a 60-minute open field study (although the authors claim a general decrease in open field spontaneous behavior for males exposed to BPA postnatally that was not corroborated by any of the above mentioned studies conducted at the same dose). The lack of effects on activity spanned doses in the order-of-magnitude range of 10^{-4} to 10^{0} mg/kg-day, including open field evaluations as well as 12-hour motor activity. Negishi et al. (2003) observed a slight statistically significant increase in immobile time (females only at 10^{1} but not 10^{0} or 10^{2} mg/kg-day) in a 12-hour automated activity test, but the lack of findings in total activity indicates that this effect had no functional consequence and cannot be regarded as an adverse effect. Taken together, BPA does not appear to have effects on horizontal activity measures.

Rearing

Rearing was measured by Aloisi et al. (2002), Dessi-Fulgheri et al. (2002), Ema et al. (2001), Farabollini et al. (1999), Kubo et al. (2001, 2003), Negishi et al. (2003, 2004), and Sato et al. (2001). The only statistically significant effect was an increase in rearing in females but not males at 10^{-2} mg/kg-day measured by Kubo et al. (2003). No statistically significant effects were noted by Kubo et al. (2003) at the higher dose of 10^{-1} mg/kg-day or at the doses ranging from 10^{-4} to 10^{-1} mg/kg-day in a much more robust study (25 litters) by Ema et al. (2001). In summary, BPA has no effect on rearing.

Grooming

Grooming was measured by Aloisi et al. (2002), Ema et al. (2001), Farabollini et al. (1999) (plus maze, holeboard), Negishi et al. (2003), and Sato et al. (2001). There was an increase in grooming measured in males only at 10^{-2} mg/kg-day (Farabollini et al. 1999) that was not

corroborated by several other studies conducted at the same dose level (Aloisi et al. 2002; Ema et al. 2001). Negishi et al. (2003) measured an increase in percentage of grooming period during 5-minute open field test at 10^0 mg/kg-day but not at 10^1 and 10^2 mg/kg-day in males. These effects are not regarded as adverse, because they represent a change from approximately 10 to 22 percent in a 5-minute period and there was no dose-response relationship. Negishi et al. (2003) speculate that the increase in self-grooming is related to increased stress, but this speculation is not supported by any experimental evidence. Thus, there was no consistent evidence of adverse effects on grooming following exposures to BPA.

Defecation

Ema et al. (2001), Farabollini et al. (1999), and Sato et al. (2001) evaluated defecation (number of fecal boli) in a very short period of 3–5 minutes during open field behavior or holeboard behavior. This endpoint is not worthy of much discussion unless there are dramatic differences. For completeness, no effects were measured at any dose (10^{-4} to 10^{-1} mg/kg-day).
A statistically significant effect was noted at 10^2 mg/kg-day, but the difference was 2.2 fecal boli in controls compared to 1.34 in BPA-treated animals (Sato et al. 2001). Therefore, there were no biologically meaningful effects on defecation following exposures to BPA.

Complex/Cognitive Behaviors

Learning and Memory

Investigators evaluated effects of BPA on active avoidance (Negishi et al. 2003, 2004), passive avoidance (Negishi et al. 2004; Kubo et al. 2001), Biel multiple T-water maze (Ema et al. 2001), and Morris water maze (Carr et al. 2003). BPA induced no consistent effects on behaviors that depend on learning and memory when comparing treated animals against their respective controls. Ema et al. (2001) did not detect effects on Biel water maze at doses spanning 10^{-4} to 10^{-1} mg/kg-day. No other studies evaluated the effects of BPA on learning and memory tests at

these lower doses of 10^{-4} to 10^{-2} mg/kg-day. Mixed results were reported in the literature at a dose of 10^{-1} mg/kg-day and higher:

- There was no significant effect on acquisition of maze solution on the Morris water maze in male or female rats exposed postnatally to 10^{-1} mg/kg-day. However there was a decrease (females only) in time spent in the escape quadrant where the platform was formerly located, indicating a possible decrease in retention of the spatial information. This possible effect on retention occurred at 0.25 mg/kg-day but not at 0.10 mg/kg-day, which are doses that are both in the same order of magnitude of 10^{-1} mg/kg-day (Carr et al. 2003). Higher doses were not tested.

- There were no statistically significant effects on single-trial passive avoidance in 13-week-old male rats (female rats not tested) at 10^{-1} mg/kg-day, although there was a tendency toward increased latency (i.e., improvement) in avoiding the shock (Negishi et al. 2004). At 10^{0} mg/kg-day, no statistically significant effects in 7-week-old males or females were reported on single-trial passive avoidance, although there was a tendency for decreased latency in males and increased latency in females compared to respective controls (Kubo et al. 2001). Higher doses were not tested.

- At 15 weeks of age, males at the 10^{-1}-mg/kg-day dose level had a decrease in acquisition of active avoidance behavior. However, there were no effects on performance during the final acquisition session or the extinction session (Negishi et al. 2004; females were not tested). This active avoidance behavior may have been confounded by prior testing of the same animals on passive avoidance. The BPA-treated animals performed better on the passive avoidance test, which may have resulted in the initial slower acquisition of active avoidance in the first three sessions. These same authors report no effects on females at 10^{0}, 10^{1}, and 10^{2} mg/kg-day (Negishi et al. 2003). At 10^{0} mg/kg-day, males performed slightly worse when they were 8 weeks old

but not 4 weeks old (Negishi et al. 2003). Inspection of the graph indicates that this decrease in avoidance occurred on the first day of testing but not on the second and third. Males performed better on active avoidance tests at 10^1 and 10^2 mg/kg-day when they were 4 weeks old, but there were no effects when they were 8 weeks old. However, there was no significant treatment x day interaction in repeated measures analysis of variance (ANOVA) at either 4 or 8 weeks of age. Based on this data and inspection of the graphs, no consistent adverse effect of BPA on active avoidance emerges.

- There were no effects in males or females on Biel water-filled multiple T-water maze at 10^{-1} mg/kg-day (Ema et al. 2001).

In summary, there were no consistent effects on learning and memory at 10^{-1}, which is a dose that was tested by several investigators. At the higher doses (10^0–10^2 mg/kg-day), there were no effects on females and no consistent effect on males across age and dose levels (Negishi et al. 2003; Negishi et al. 2004; Kubo et al. 2001).

In comparing the different results, it is important to recognize that different types of learning and memory tests were used. Although it can be argued that different tests measure different aspects of learning and memory, and that none of these methods are sensitive to all types of effects on learning and memory, there are some methods that would generally be regarded as better than others. The Morris water maze, as described by Carr et al. (2003), appeared to be the most thorough controlled evaluation of learning and memory compared to the other methods used for the behavioral tests (active avoidance, passive avoidance, and possibly the Biel maze). The active avoidance test evaluated acquisition over a 3-day period (Negishi et al. 2003, 2004), which means there was a learning component to the test. In Negishi et al. (2004), the active avoidance test may have been confounded by the passive avoidance test that was performed in the same animals two weeks earlier. The passive avoidance test was a one-trial test that did not have an acquisition component (Negishi et al. 2004; Kubo et al. 2001) and is the least reliable test that does not have a learning component. The one-trial passive avoidance test can be highly confounded by the activity level of animals. The Biel water maze was not described adequately

(Ema et al. 2001), but typically, this test evaluates both acquisition and retention and would be considered a more robust evaluation than the one-trial passive avoidance test.

A second equally important consideration in evaluating the methods is the sample size and whether the litter was considered the experimental unit. Only Negishi et al. (2004) and Ema et al. (2001) considered the litter as the experimental unit. This is assumed to be true for Negishi et al. (2003), even though it is not clearly specified. The study conducted by Ema et al. (2001) tested six rats (each from a different litter/sex/group) on the Biel maze. The studies by Negishi et al. (2003, 2004) used as many as 8–10 litters/group with 1 pup/litter tested. Carr et al. (2003) dosed 10 pups/sex/dose group directly from postnatal day (PND) 1–14 from an unspecified number of litters. Because the pups were dosed so early, at an age when they are completely dependent on the dam, it would have been preferable for the litter to be the experimental unit. Kubo et al. (2001) tested 11–14 pups/dose group, but did not describe how these pups were selected from the five litters that were dosed through the dam. This study should have evaluated the data using the litter as the statistical unit.

Taken together, there is no consistent evidence that BPA causes adverse effects on learning and memory. Some of these studies would need to be repeated using sufficient sample size representing different litters before definitive conclusions can be reached.

Carr et al. (2003) and Kubo et al. (2001) claim that BPA disrupted normal gender differences on Morris water maze and passive avoidance, respectively. As discussed later in the section devoted to evaluating effects of BPA on sexual differences in behavior, there is conflicting evidence and the results are not compelling

In summary, there was no consistent evidence indicating that BPA causes adverse effects on learning and memory as measured by the behavioral tests conducted.

Operant Behavior for Delayed Larger Reinforcement

BPA (10^{-2} mg/kg-day) improved performance on a complex operant behavior involving a choice of two nose-poking holes (Adriani et al. 2003). One nose-poke hole provided immediate and small reinforcement (IAS). A second nose-poke hole provided delayed and larger reinforcement (LAD), but the delay was increased gradually. Nose-poking (in either hole) during this delay was considered "inadequate nose poking" but was not punished. It should be noted that there were several inconsistencies between the figures and the text. This analysis assumes that the text is correct and the figures were mislabeled. The authors concluded that BPA-treated rats of both sexes showed a marked preference for the LAD hole during the whole experiment, indicating decreased impulsivity. In addition, as the delay was increased for the preferred LAD hole, the BPA-treated male rats exhibited reduced inadequate responding compared to controls. There were no effects of BPA on inadequate responding in females compared to controls. The authors concluded that BPA decreased impulsivity, which is contrary to what they initially hypothesized based on other literature. Functionally, BPA increased efficiency in response by reducing responses that had no consequence, and increased responses that led to substantial increase in reinforcement. Thus, this effect, by itself, cannot be considered an adverse effect.

The authors also interpreted the decrease in inadequate nose poking in males as evidence of feminization of the brain. This conclusion is not supported by the experimental results and is also not consistent with the hypothesis that estrogenic chemicals will masculinize the developing male brain. The use of terms such as feminization or masculinization implies a mode of action that involves disruption of normal estrogenic or androgenic effects during development of the brain. Such terms should not be used unless there is experimental evidence to support a link of this mode of action to the behavior being evaluated. In addition, the inadequate responding had no scheduled consequences. Behaviors that are not under schedule control can be much more variable. Therefore, it becomes necessary to demonstrate that the sex difference in this behavior is reproducible and responsive to hormonal effects before a hypothesis regarding feminization or masculinization can be proposed. Finally, this effect on inadequate nose poke must be evaluated within the context of the finding that BPA increased nose-poking in both males and females in the hole associated with delayed but larger

reinforcement. There were no sex differences in this schedule-controlled behavior in untreated animals or in BPA's effect on this behavior.

The results of this study can also be interpreted in the context of the cognitive effects of BPA discussed in the previous section. This study indicates that BPA improved the ability of rats to perform a complex operant task that is dependent on learning and memory. These results provide additional supporting evidence that BPA does not adversely affect cognitive behaviors.

Complex/Emotional Behaviors

Several authors evaluated complex behaviors that they related to feelings of impulsivity and anxiety. There was a tendency to anthropomorphize behaviors, as discussed above. However, for the purposes of this discussion, the conclusions of the authors are reported.

Impulsivity

As discussed immediately above, BPA at a dose of 10^{-2} mg/kg-day reduced impulsivity, as measured by a complex operant behavior involving nose poking for delayed higher reinforcement (Adriani et al. 2003). This effect, by itself, is not an adverse effect. These results need to be replicated before definitive conclusions can be made.

Stress and Anxiety

Three studies measured behaviors that the authors related to stress and/or anxiety (Adriani et al. 2003; Farabollini et al. 1999; Sashihara et al. 2001). Sashihara et al. (2001) measured an increase in distress vocalization, but no effects on several other parameters, in chickens that were isolated and stressed, according to the authors. In this study, BPA was injected by intracerebral injection, and no data were provided that relate these doses to the more relevant oral route of exposure. No conclusions can be made regarding effects of BPA on humans.

Adriani et al. (2003) measured the effect of 10^{-2} mg/kg-day of BPA on time spent in a novel chamber that was connected with a familiar chamber. Although there was no effect on treated males on time spent in the novel environment, the authors concluded that BPA was associated with more marked levels of "novelty-induced hyperactivity." The authors suggested that "novelty-induced hyperactivity" might be an indication of increased stress due to the novel environment. This suggestion is speculative, and there is no evidence that the increase in activity in the novel chamber is an adverse effect. An equally valid anthropomorphic interpretation is that BPA increased exploration while in a novel environment, which is an adaptive response. In contrast, Farabollini et al. (1999) concluded that BPA (10^{-1}, 10^{-2} mg/kg-day) reduced anxiety, as measured by behavior on an elevated plus maze, including more frequent entry into the open arm compared to the closed arm. Finally, Negishi et al. (2004) found no effects of BPA at 10^{-1} mg/kg-day on the number of entries into the open compared to the closed arm in the elevated plus maze. Thus, there were no consistent effects to indicate that BPA affects anxiety as measured by activity on the elevated plus maze.

Pain-Related Behaviors

One study by Aloisi et al. (2002) evaluated the effect of BPA (10^{-2} mg/kg-day) on phasic painful stimulation. The formalin test was conducted by subcutaneous injection of a dilute solution of formalin. This induced a series of behavioral responses (licking, flexing, and jerking of the injected hind limb).

The underlying hypothesis is that estrogens modulate the opioid system, which in turn, affects perception of pain. Aloisi et al. (2002) reported that in a previous study, estradiol and progesterone reduced nociception in male rats as measured by a formalin test, and intracerebroventricular administration of estradiol affected formalin-induced behavioral responses in adult male rats. This latter effect was reversed by pre-treatment with naloxone (an aspecific opioid antagonist). Aloisi et al. (2002) reported that prenatal BPA treatment induced an increase in licking duration in females (not statistically significant) and in flexing duration in both sexes in the first half of the test (0–30 minutes after formalin injection). They also reported that postnatal BPA treatment induced a decrease in paw-jerk frequency in males and

females during the second part of the test (30–60 minutes after formalin injection). There were no effects on plasma concentrations of corticosterone, estradiol, and testosterone. There were no statistically significant differences in spontaneous behaviors (exploration, internal crossing, external crossing, rearing, grooming) between the formalin and sham tested groups.

Further experiments are needed to reproduce the results with adequate litter size before any definitive conclusions can be made. The relevance of these effects to humans requires an evaluation of the validation of this animal model, which was beyond the scope of this review.

Social Behaviors

The studies of Dessi-Fulgheri et al. (2002) and Farabollini et al. (1999, 2002) are frequently cited as evidence that BPA affects social play behavior, sociosexual behavior, and non-social behaviors, respectively. In addition, Kawai et al. (2003) concluded that BPA increased aggressive behavior, and Palanza et al. (2002) concluded that BPA adversely affects maternal behaviors. These results have not been replicated in other laboratories or by the investigators themselves. These studies are discussed because they appear to be misinterpreted as providing strong experimental data indicating that BPA causes adverse effects in behaviors that are directly relevant to human behaviors.

Social Play Behavior

Dessi-Fulgheri et al. (2002) observed effects of BPA (10^{-1}, 10^{-2} mg/kg-day) on play behaviors directed to females, low-intensity mating elements, sociosexual exploration, and social interest. These behavioral factors were defined by the principal component method of statistical analysis of the many individual behavioral measures. Once these behaviors were grouped statistically into different factors, the authors named these clusters of behaviors as play behavior, defensive behavior, social interest, etc. These classifications are highly subjective and are established by definition and not by any experimental evidence. The authors then combined the frequencies of all the behaviors linked together by the factor analyses and conducted an ANOVA. Based on this second layer of statistical analysis, the authors concluded that BPA caused masculinization

of female behavior in two behaviors (play with females and sociosexual exploration), and an intensification of male play behavior toward females.

The conclusions of this paper depend on multiple layers of statistical assumptions that ANOVA of pooled factors from principal component analyses is appropriate. There is also a circular argument used in claiming that the statistical analyses demonstrate effects on behaviors that were basically defined by the authors. Even if one were to give the authors the benefit of the doubt regarding their opinion about what these different clusters of behaviors represent, the principal component method is just a statistical tool to generate a hypothesis about the relationships among variables within a study. A separate study must be conducted to determine whether the principal component method results in similar factor loadings. Until this validation is conducted, it is inappropriate to generalize these results.

From a purely behavioral science research perspective, this paper provides an interesting approach to analyzing data and using statistics to identify structure and relationships among the different behavioral parameters. This is preferable to evaluating statistical significance on individual behavioral measures in isolation. However, such an approach requires rigorous validation that is lacking in these studies. From a regulatory science perspective, this paper does not provide sufficient evidence that BPA causes adverse effects on play behavior or masculinization of female behavior.

Sociosexual Behavior

Farabollini et al. (2002) and Kubo et al. (2003) studied the effects of perinatal exposure to BPA (10^{-2} and 10^{-1} mg/kg-day) on sociosexual behavior. These behaviors are outside the scope of this review on the effects of BPA on neurobehavioral endpoints. Therefore, the evaluation of this study may be out of context of other papers that may report effects of BPA on sociosexual behavior.

Kubo et al. (2003) reported that BPA (10^{-2} and 10^{-1} mg/kg-day) had no effects on male or female sexual behavior, while diethylstilbestrol (DES) and *trans*-resveratrol (RVT) reduced

female receptivity to males. Farabollini et al. (2002) conclude that on the whole, male sexual behavior was not disrupted, and there was a slight intensification of female sexual behavior. They state that these effects are not consistent with the hypothesis that BPA masculinizes the brain. Farabollini et al. (2002) concluded that even slight changes in the sphere of sexual behavior may have important consequences in terms of fitness and welfare at the individual level, with consequences on population dynamics. This conclusion is not supported by the experimental evidence from both of these studies taken together. In addition, the slight effects reported by Farabollini et al. (2002) have not been demonstrated to decrease reproductive function, as evidenced by the lack of findings at low dose levels in two large multi-generation reproduction studies (Ema et al. 2001; Tyl et al. 2002).

Non-Social Behaviors

The results of Farabollini et al. (1999) are summarized briefly above in the discussion on BPA (10^{-2}, 10^{-1} mg/kg-day) effects on anxiety. In the aforementioned discussion, we accepted the authors' conclusion that BPA reduces anxiety in males. The authors also concluded that BPA decreased motor activity parameters, as well as the motivation to explore in females and males. This paper is often cited as providing evidence that BPA permanently influenced neural systems involved in the organization of behavior. For this reason, it is important to discuss this paper in some detail. First, there were no neuroanatomic or neuropharmacologic data to indicate that BPA specifically influences neural systems. Second, the magnitude of the statistically significant effects (control vs. dose group) appears to be quite small. For example, the number of entries into various sections (2.5 vs. 0.9 open-arm entries; 2.5 vs. 4.2 closed-arm entries; all comparisons are control vs. a treated group), number of stretched-attend postures (2.6 vs. 4.6), and frequency of head-dipping (2.4 vs. 5.8) are of questionable biological significance and cannot be interpreted as adverse effects in and of themselves (control vs. a treated group). Third, this is largely a negative study, considering the multiple comparisons made on many behavioral endpoints, some of which are mathematically related (frequency and duration of same behavior; or ratios of behavioral endpoints). For example, in low-dose males, increases in open-arm entries, percent time in open arms, and percent open/total entries and a decrease in stretched-attend posture were the only statistically significant findings. Yet three of these

behaviors are really the same measure expressed mathematically in different ways. Fourth, the authors appear to use a circular argument in using the results of the principal component analyses to identify other behaviors as measures of "anxiety" or "motivation to explore." For example, the factor analyses linked head dips with stretched-attend. Because the investigators believe that stretched-attend behavior is an indicator of anxiety, they concluded that the frequency of head dips must also be related to "anxiety." There is no evidence that any of the statistically significant effects have any functional significance or can be related to how the animal feels (e.g., anxious, motivated to explore). In conclusion, this paper identifies relatively few statistically significant effects on behavioral parameters that have not been demonstrated to cause adverse functional effects.

Aggression

Kawai et al. (2003) concluded that the results of their study "demonstrate that bisphenol A temporarily activated aggressive behavior in mice at 8 weeks of age and that low doses of bisphenol A interfered with the normal development of reproductive organs." However, Kawai et al. (2003) evaluated cumulative contact time with a control rat at BPA doses of 10^{-2} and 10^{-3} mg/kg-day and assumed that cumulative contact time is a reliable measure of aggression, even though the mice sniffed intruders but did not attack. The biological significance of a difference of 30 seconds of contact time (approximately 20 seconds in controls vs. 45–50 seconds in treated animals) cannot be determined without evaluating historical control data. In addition, there were no effects seen at 12 or 16 weeks of age, and both doses of 10^{-2} and 10^{-3} mg/kg-day gave the same magnitude of effects at 8 weeks. In contrast, Dessi-Fulgheri et al. (2002) reported that BPA (10^{-2} mg/kg-day) had no effect on aggressive behavior (offensive behavior) in an intruder test that was conducted more rigorously by distinguishing between behaviors that were offensive, defensive, and ambivalent.

Taken together, these results do not indicate that BPA has a treatment-related effect on aggressive behavior.

Maternal Behavior

Palanza et al. (2002) is the only study included in this review that evaluated maternal behaviors of females from the first generation (F1). In this study, pregnant females were fed daily doses of corn oil (controls) or 10^{-2} mg/kg-day of BPA during gestation days 14–18. As adults, the prenatally treated female offspring were time mated and again fed either corn oil (controls) or the same doses of BPA on gestation days 14–18, resulting in four treatment groups: controls, prenatal BPA exposure (BPA-oil), adult BPA exposure (oil-BPA), and both prenatal and adult BPA exposure (BPA-BPA). Several normal behaviors of dams were recorded. These behaviors included 1) dam was in nest, 2) nursing, 3) licking pups, 4) nest-building, 5) eating/drinking, 6) grooming, 7) moving about cage, 8) resting outside of nest, and 9) suckled by pups that dam was avoiding. Based on these behaviors, two additional categories were created and statistically analyzed: nest-related behaviors (sum of incidence of nursing, nest building, and in-nest activity) and out-of-nest behaviors (sum of incidence of active, eating/drinking, grooming, resting).

In the BPA-oil and oil-BPA treated dams, there were decreases in percent incidence of normal maternal behaviors related to nesting, but no effects were observed in the BPA-BPA group. Although the authors concluded that BPA affected maternal behaviors, it is important to emphasize that none of the behaviors measured were considered aberrant, and the range of acceptable incidence of these behaviors in normal controls was not discussed. These statistically significant effects in incidence of maternal behaviors had no impact on the functional developmental of the pups as measured by growth and ontogeny of cliff avoidance and righting reflexes. BPA has also been demonstrated to have no effect on developmental and reproduction endpoints in multi-generation studies following exposures to 10^{-4} to 10^{0} mg/kg-day (Ema et al. 2001; Tyl et al. 2002). The experimental data do not support the author's conclusion that BPA causes "adverse" effects on maternal behaviors.

Pharmacologic Challenge and Psychological Dependence

Four papers evaluated the effect of BPA exposures on pharmacologically induced behaviors. The underlying hypothesis being tested is that alteration of a pharmacologically induced behavior could indicate that developmental exposures to BPA have effects on the developing nervous system at the receptor level. These effects may have functional consequences in humans that are not detectable in animal models except by evaluating changes in the functional response to a pharmacologic agent.

Adriani et al. (2003) reported that perinatal exposures to BPA (10^{-2} mg/kg-day) attenuated the activity-increasing effects of 1 mg/kg amphetamine in males but not in females. (Amphetamine increases dopamine and norepinephrine levels in the synapse.) This study is limited by the fact that the control group (1 mg/kg amphetamine in the absence of BPA) did not cause an increase in activity in females, which raises some questions about the reliability of the probe dose. Specifically, amphetamine's effects on motor activity are biphasic, and 1 mg/kg is a relatively high dose that could begin to cause decreases in horizontal activity. The results of this study raise the hypothesis that BPA attenuates amphetamine-induced increased activity. It is difficult to make firm conclusions based on a single probe dose of amphetamine.

Negishi et al. (2004) evaluated the effects of perinatal exposure to BPA (10^{-1} mg/kg-day) on activity-increasing effects of 5 mg/kg tranylcypromine (Tcy), a monoamine oxidase inhibitor. Monoamine oxidase inhibitors inhibit the metabolism of norepinephrine, dopamine, and serotonin (5HT), resulting in an increase of these neurotransmitters in the synapse. BPA attenuated the Tcy-induced increase in activity level. The results of Negishi et al. (2004) are consistent with those reported by Adriani et al. (2003). Both studies report that BPA attenuated the activity-increasing effects of a probe dose of pharmacologic agents that increase levels of dopamine and norepinephrine in the synapse.

Suzuki et al. (2003) reported that prenatal and neonatal exposure to BPA in the diet (roughly estimated to be 10^{-2}, 10^{1}, and 10^{2} mg/kg-day; see Appendix 1) enhanced the pharmacologic actions of the psychostimulant methamphetamine in males (METH, 2 mg/kg s.c.; female offspring were not tested). The pharmacologic mechanism of METH is similar to that of

amphetamine. Thus, this enhancement is not consistent with the attenuating effects of amphetamine and Tcy that were reported by Adriani et al. (2003) or Negishi et al. (2004).

Suzuki et al. (2003) also demonstrated that BPA can potentiate the effects of METH in a different behavioral paradigm. They evaluated the effects of BPA on a conditioned place preference procedure that paired METH with placement in one chamber (METH-chamber) and saline with placement in a different chamber (SAL-chamber). BPA (10^{-2}, 10^{1}, and 10^{2} mg/kg-day) enhanced the dopamine D1 receptor-dependent preference for the METH-chamber. In addition, BPA (10^{2} mg/kg-day) significantly shifted the dose-response curve of METH to the left. Thus, the potentiating effect of BPA was more reliably demonstrated than other laboratories that typically use a single probe dose. These investigators also provided preliminary evidence that perinatal BPA (10^{2} mg/kg-day) exposure produced a significant increase in the dopamine D1 receptor production in the whole brain. These neuropharmacologic effects are consistent with a hypothesis that chronic BPA may cause supersensitivity of METH effects through the mode of action of upregulation of dopamine D1 receptor function. Although this study was better than most papers, in that a possible mode of action for behavioral effects was investigated, it has two important limitations: 1) the litter was not the experimental unit, and the number of dams dosed was not reported, and 2) concentrations in food were reported but authors did not report chemical consumption.

Suzuki's laboratory conducted a similar separate experiment to evaluate the effects of BPA (roughly estimated to be 10^{-2}, 10^{1}, and 10^{2} mg/kg-day; see Appendix 1) on morphine-induced effects on locomotion and conditioned placement (Mizuo et al. 2004). All doses of BPA potentiated the hyperlocomotion and conditioned preference of morphine, although only the two higher doses of BPA (10^{1}, 10^{2} mg/kg-day) were statistically significant. The authors cited other literature indicating that morphine-induced place preference can be blocked by dopamine antagonists. This suggests that dopamine-containing neurons in the midbrain, which has a high density of opioid receptors, could play a critical role in the rewarding effects and hyperlocomotion of opiates like morphine. The investigators determined experimentally that perinatal exposure to BPA (10^{2} mg/kg-day) did not appear to upregulate opiate receptors. Therefore, based on the results of Suzuki et al. (2003), these investigators hypothesized that the

mode of action for BPA's potentiation of the behavioral effects of morphine is the result of direct effects on the central dopaminergic system.

At present, it is premature to make conclusions about the human relevance of these pharmacologic challenge studies until they can be repeated by other laboratories using adequate sample size. For some perspective about dose levels, the doses at which effects were more reliably demonstrated were at dietary concentrations of 2 mg BPA per gram of diet (highest dose level). Assuming that an adult, 100-gram mouse will eat approximately 15 grams of food (150 gram diet/kg), the high dose is roughly equivalent to 300 mg BPA/kg body weight. The estimate of the amount of food an adult mouse eats is based on data from the Animal Care and Use Committee of the University of Iowa (http://research.uiowa.edu/animal/?get=mouse). The doses at which some effects were detected were 10^{-2}, 10^{1}, and 10^{2} mg/kg-day, with more consistent evaluations reported at 10^{2} mg/kg-day.

There are two reasons why these papers are of interest. First, they provide preliminary pharmacologic and behavioral evidence that supports a potential mode of action of upregulating dopamine receptors. Second, the behavioral paradigm has been used in psychopharmacology to study drug preference, so these results have the potential of being misinterpreted as providing definitive evidence that exposures to BPA will enhance the psychological dependence of psychomotor stimulants, as suggested by the authors. This last conclusion is highly speculative based on the existing experimental evidence.

In conclusion, there are conflicting reports on the effects of lower doses of BPA on behavioral effects following pharmacologic challenge to drugs that increase levels of dopamine and norepinephrine in the synapse. It is premature to make definitive conclusions regarding the effect of higher doses of BPA (10^{2} mg/kg-day) based on results from one laboratory.

Brain Structure

Kubo et al. (2001, 2003) reported that BPA (10^{-2}, 10^{-1} and 10^{0} mg/kg-day) reduced the sexual differences in volume and number of neurons of the locus coeruleus in males and females but

did not alter the sexually dimorphic nucleus of the preoptic area (SDN-POA). The authors reported that the locus coeruleus is larger in control females than in males, but that BPA eliminated or reversed the sexual difference. In other words, BPA exposure tended to increase the size of the locus coeruleus in males and decrease the size in females. These results are of potential concern, because they suggest an effect on neuronal developmental processes. In addition, diethylstilbestrol (DES, 6.5 μg/kg-day), a synthetic estrogen, had similar effects. The lack of effects of DES on the SDN-POA is somewhat surprising, especially given that these authors originally included DES as a positive control. The authors hypothesize that this may have been due to using lower doses of DES than those used by other investigators. These results need to be evaluated more rigorously for consistency with the larger literature on the effects of estrogenic chemicals such as DES on sexually dimorphic nuclei (e.g., compare relative sensitivity of locus coeruleus and SDN-POA to DES). This is beyond the scope of this review.

The results of Kubo et al. (2001, 2003) are currently based on relatively small sample sizes of 6–7 pups from 5 litters or 7–8 pups from 6 litters. The selection of these animals from the 5–6 litters per group was not described, and the individual animal was the statistical unit of analysis (Student's t-test without correction for multiple comparisons). The sex difference in volume and cell count of locus coeruleus is relatively small compared to SDN-POA. In addition, the effects of BPA are relatively small (approximately 15–20 percent). Historical control data from the laboratory will aid in understanding normal variation in size and cell number. Therefore, these results should be considered preliminary findings that need to be repeated in a larger study with the litter as the experimental unit, with identical processing of the brain to control shrinkage or expansion of tissues, and with the morphologic measurements conducted without knowledge of treatment level. No definitive conclusions can be made based on these preliminary results from a single laboratory.

Reduction of Sexual Differences in Behavior ("Masculinization" of Females and "Feminization" of Males)

There were no consistent effects indicating that BPA reduces sexual difference in behaviors. In many cases, the reported analyses relied on subjective evaluation of BPA effects that are so slight that they do not result in statistically significant effects on BPA-treated males and females when compared to their respective controls. This section focuses on those studies that report an effect of BPA on sexual differences in behavior. These same papers are analyzed more critically in Appendix 1 and earlier sections of this report. For the purposes of this section, the conclusions of the authors are presented. There is a need for more rigorous use of the terms masculinization and feminization, especially when discussed as conclusions instead of hypotheses that require further testing. The use of terms such as feminization or masculinization implies a mode of action that involves disruption of normal estrogenic effects during development of the brain. Such terms should not be used unless there is evidence to support this mode of action. There was very little reliable validation that chemicals with estrogenic effects would have a similar effect on behaviors.

Sex Difference in Activity

Kubo et al. (2001, 2003) reported that BPA (10^{-2}, 10^{-1} and 10^{0} mg/kg-day) decreased the sexual differentiation of open-field behavior for a period of 10 minutes. At the 10^{-2}, 10^{-1} mg/kg-day dose level, 20–24 rats/group were tested, but there was no clear explanation of how these animals were selected from the five or six litters/dose group. The basis for this conclusion is that activity in control females is statistically significantly higher than in control males. Following exposure to BPA, there are no longer statistically significant differences between male and female activity in the open field. Thus, even though BPA had no statistically significant effects on activity in males and females when compared to their respective controls, these authors consider the lack of statistically significant difference in BPA-treated males and females as evidence of decreased sexual differentiation. These effects are not sufficiently robust to be considered conclusive evidence. Confirmation of this effect could have been determined by evaluating the results of Ema et al. (2001). Unfortunately, no data were shown because there

were no effects of BPA on open field activity. Statistical analyses (two-way ANOVA with sex and treatment as factors) of rearing and number of crosses on a holeboard (open field with four holes in the floor) indicated that BPA (10^{-1}, 10^{-2} mg/kg-day) resulted in no sex × treatment effect or sex effect (Farabollini et al. 1999). These results are not consistent with the results of Kubo et al. (2001, 2003). Negishi et al. (2003) evaluated higher doses of BPA (4, 40, 400 mg/kg-day) and reported that the ANOVA for gender revealed no significant effect on sex difference for motor activity.

Sex Difference in Learning and Memory

Kubo et al. (2001) also reported a decrease in sexual difference in passive avoidance following perinatal exposures to BPA at 10^{0} mg/kg-day. They reported that control males perform better (have higher latency) on passive avoidance when compared to control females. These gender differences were statistically significant. Following BPA exposure, the gender differences were not statistically significant. BPA tended to reduce latency in males and increase latency in females (no statistics reported). In other words, males performed worse following exposures to BPA. The authors did not repeat this test in a subsequent paper (Kubo et al. 2003). Negishi et al. (2004) evaluated the effects of BPA (10^{-1} mg/kg-day) on passive avoidance but evaluated only males. Although females were not evaluated, this study is relevant because Negishi reported a non-statistically significant *increase* in latency in males (i.e., performed better). This is an opposite effect to that reported by Kubo et al. (2001). Negishi evaluated one pup/litter from eight or nine litters. Kubo evaluated 11–14 pups/group from five litters and did not report how many pups were selected per litter. These results indicate that the behavioral effects on passive avoidance in males could not be replicated in another study using twice as many litters.

Carr et al. (2003) concluded that a low BPA dose (0.1 mg/kg-day) disrupted normal gender difference in acquisition, whereas a higher BPA dose (0.25 mg/kg-day) had no effect. However, the difference in doses used is not sufficient to be regarded as "low" or "high" as the authors indicate. The lack of effect at 0.25 mg/kg-day does not corroborate the effects noted at 0.1 mg/kg-day. In addition, there were no statistically significant gender differences in control animals on the first three days of acquisition following exposure to 0.1 mg/kg-day BPA, and

neither 0.25 nor 0.1 mg/kg-day BPA negatively affected acquisition in either males or females. Thus, the data is not sufficiently robust to support a conclusion that low dose BPA reduces sex difference.

Negishi et al. (2003) reported no significant effect of BPA (10^0, 10^1, 10^2 mg/kg-day) on sex difference in the active avoidance test.

Thus, there is conflicting evidence regarding the effects of BPA in reducing sexual difference in the different learning and memory tests. At present, these results are not compelling.

Sex Difference in Nonsocial, Social, and Sexual Behaviors

Farabollini et al. (1999) evaluated several non-social behaviors on holeboard and elevated plus-maze test. They concluded that "contrary to our expectation, a clear masculinization of females was not observed." Dessi-Fulgheri et al. (2002) observed sex differences in four behavior categories (play with females, low-intensity sexual behavior, sociosexual exploration, and ground exploration). Of these behaviors, they reported that BPA masculinized female behavior in two of the categories (play with females and sociosexual exploration) but not in ground exploration or low-intensity sexual behavior. Farabollini et al. (2002) reported that BPA (10^{-2} mg/kg-day) "did not masculinize female behavior or potentiate masculinization processes of males." On the contrary, they reported a "potentiation of female behavior in females and a depotentiation of male behavior in males." These studies are discussed in greater detail in a previous section and in Appendix 1.

Discussion/Conclusions

A review of the recent literature on the effects of BPA on neurobehavioral endpoints was conducted based on a literature search performed by the American Plastics Council. This review considered 18 studies published between 1999 and 2004 that included neurobehavioral endpoints.

The studies evaluated the potential effects of BPA on a wide range of different behavioral paradigms and statistically analyzed many individual behavioral measures. This review focused on evaluating the consistency of effects on general behavioral endpoints. There was no consistent evidence that low-dose BPA (e.g., below 50 mg/kg-day) caused adverse effects on behavioral endpoints, including 1) ontogeny of sensory/motor behaviors and reflexes; 2) frequently measured gross behaviors of ambulation, rearing, grooming, and defecation; 3) complex cognitive behaviors (Morris water maze, Biel maze, active avoidance, passive avoidance, and operant behavior); 4) complex emotional behaviors such as impulsivity, anxiety, and pain perception; and 5) social behaviors such as play, aggression, maternal behavior, and sexual behavior.

A few investigators suggest that statistically significant effects in one or the other direction indicate that BPA causes masculinization of female behavior or feminization of male behavior on individual measures. However, there is no convincing or consistent pattern of effect on behavior that supports this conclusion.

There is preliminary evidence that BPA may potentiate behavioral effects of drugs that are known to increase catecholamine levels in the synapse. In addition, prenatal exposure to BPA induced neuropharmacologic effects that suggested upregulation of dopamine receptor. This mode of action is consistent with the potentiation of the pharmacologic effects on behavior and would be considered relevant to humans because it suggests longer-lasting effects on the development of the nervous system at the receptor level. There are conflicting reports on the effects of lower BPA doses on behavioral effects following pharmacologic challenge to drugs that increase levels of dopamine and norepinephrine in the synapse. The effects at higher doses

of BPA (10^2 mg/kg-day) are based on results from one laboratory using unreported numbers of litters.

There is also preliminary evidence from one laboratory that perinatal exposure to low doses of BPA (10^{-2}, 10^{-1}, 10^0 mg/kg-day) reduced the sexual difference in size of the locus coeruleus, which is a nucleus in the midbrain related to the central noradrenergic system in the brain. There were no effects noted on the sexually dimorphic nucleus of the preoptic area in the hypothalamus. These effects need to be evaluated for consistency within the larger literature on the effects of estrogenic chemicals on these brain areas.

These preliminary pharmacologic and morphologic effects suggest a potential developmental effect of BPA on the structure and receptor function of the brain. It should be emphasized that it is premature to reach a definitive conclusion as to whether these effects are relevant adverse effects until these findings are replicated and compared to historical control data. Reproducibility of results in independent laboratories under well-controlled experimental conditions and adequate sample size is an essential part of scientific investigation to test a hypothesis.

Many of the behavioral effects need to be replicated as well. At present, there are a number of fragmentary results but no consistent overall pattern of effects. Several of the behavioral paradigms are novel tests that are not widely used, or the behavioral tests (e.g., one-trial passive avoidance) are known to produce variable response in control animals. These tests need to be validated using positive or negative controls (dose response and not just a single high, near-lethal dose) to demonstrate that the behavior is measuring the effects claimed. Historical control data from the laboratories on different behavioral parameters are also needed to understand the normal range of measurements. Much more rigorous experimental evidence is needed before terms such as "masculinization" and "feminization" can be used with any confidence to describe behavioral or morphological effects.

In conclusion, there are no consistent adverse effects of perinatal exposures to low doses of BPA on neurobehavioral endpoints based on the 18 studies that were reviewed.

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Table 1. Summary of Bisphenol A (BPA) Neurobehavioral Studies

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve, – = decrease or impair, 0 = no change)	Effect of Treatment; Female (+ = increase or improve, – = decrease or impair, 0 = no change)	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Adriani, 2003 Altered profiles of spontaneous novelty seeking, impulsive behavior, and response to D-amphetamine in rats perinatally exposed to bisphenol A	Sprague-Dawley rat dams	9 dams/group	40 μg/kg-day (0.04 mg/kg-day)	oral micropipette; dams from mating to weaning (GD 0–PND 25)	no, behavior scored from video recording	1 rat/sex/litter	PND 30–45; different groups counterbalanced over time	Novelty preference test 2-chamber Day 1–3 familiarization phase; Day 4 novelty preference test	time in novel environment	0	–	F<M	no	Both gender controls spent >50% of time in novel environment by end of session; BPA males matched controls; BPA females spent less time in novel environment.
									activity level in novel environment (# line crossings with both forepaws scored)	+	+	yes; statistically significant sex by time interaction	no	BPA increases activity rate in the novel environment; authors suggest this is index of novelty-induced stress
					automatic	1 rat/sex/litter	>PND 70 and 1 week after impulsivity test	Impulsivity test operant 2-nose poking holes. Training phase 1 week, testing phase 1 week.	% choice immediate and small hole (IAS)	–	–	no	not reported	BPA-treated animals were associated with a more marked preference for the LAD reinforcer and reduced inadequate responding in males
									% choice large and delayed hole (LAD)	+	+	not reported	not reported	
									frequency of inadequate nose poking (occurring during delay between LAD nose poke and delivery of five pellets)	–	0	M>F	M=F (males decrease inadequate nose poke)	
					yes video recorded	1 rat/sex/litter; however 4 litters used for saline and 5 for amphetamine (as per Figure 4 of paper)	>PND 70 and 1 week after impulsivity test	Open field with pharmacologic challenge with 1 mg/kg amphetamine	crossing	–	0	no, based on graphs	n/a	Figure 4 shows no statistically significant increase in activity following exposure to amphetamine in control groups. This questions sensitivity of test to detect amphetamine increase for untreated animals. 4–5 animals may not be sufficient for motor activity. Figure 4 also shows no effect of BPA on activity levels on saline treated animals.
									rearing	–	0	no based on graphs	n/a	
									grooming	measured	not reported	not reported	n/a	

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve; – = decrease or impair; 0 = no change)	Effect of Treatment; Female (+ = increase or improve; – = decrease or impair; 0 = no change)	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Aloisi, 2002 Exposure to the estrogenic pollutant bisphenol A affects pain behavior induced by subcutaneous formalin injection in male and female rats	Sprague-Dawley rat dams	13 control dams 7 BPA dams Pups from these dams were cross-fostered to study differences between pre-natal from post-natal exposures	40 μg/kg-day (0.04 mg/kg-day)	oral pipette; GD0 "during pregnancy and lactation"										
				BPA-prenatal (placental route): offspring of dams receiving BPA but suckled by controls	no	11 male pups and 9 female pups	22 weeks	60-min open field formalin test	licking duration, flexing duration, paw-jerk frequency	0,+,0	0,+,0 (authors claim non-statistical increase in licking)	not stated	n/a	Statistical significance is based on analysis on sub-periods of 0–5, 15–40, 40–60 minutes.
								60-min open field spontaneous behaviors following formalin injection	exploration, internal crossing, external crossing, rearing, grooming	0 (authors claim general decrease but not supported by statistics)	0 (authors claim general increase but not supported by statistics)	not stated	authors claim yes, but not supported by data	Table 2: no statistically significant group (BPA) effect, sex x group effect, group x formalin (sham vs formalin) effect, or sex x group x formalin effect. Behaviors should not be evaluated in isolation. Decrease rearing can be compensated by increase crossings.
								hormone levels	corticosterone, estradiol, testosterone	0	0	yes	no	
				BPA-postnatal (suckling route) offspring of mothers receiving oil but suckled by mothers receiving BPA	no	11 male pups and 9 female pups	22 weeks	60-min open field formalin test	licking duration, flexing duration, paw-jerk frequency	0,0,–	0,0,–	not stated	n/a	
								60-min open field spontaneous behaviors following formalin injection	exploration, internal crossing, external crossing, rearing, grooming	(-) (authors claim general decrease, this is better than other claims made)	0 (authors claim general increase but not supported by statistics)	not stated	n/a	As stated above, there were no group, sex x group, group x formalin, or sex x group x formalin effects. There was a consistent pattern of decrease in all activity measures in males but not in females.
								hormone levels	corticosterone, estradiol, testosterone	0, (+),0	0, (+), 0	yes	no	These results need to be considered relative to BPA literature on hormone levels

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve – = decrease or impair 0 = no change)	Effect of Treatment; Female (+ = increase or improve – = decrease or impair 0 = no change)	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Carr, 2003 Effect of neonatal rat bisphenol A exposure on performance in the Morris water maze	Fischer 344 rat pups	10/sex/dose group A within litter design was used such that there was a member of each treatment group for each sex in each litter. No report of total number of litters	100 μg/kg/day (low) 250 μg/kg/day (high) (0.1 and 0.25 mg/kg-day) 72 mg/kg 17β-estradiol (E2)	gavage; PND 1–PND 14 dosed between 1300 and 1600 hr daily	no	10/sex/group	weighed daily	Weight	body weight	0 E2 0 BPA	0 E2 0 BPA	not stated		Data not shown
					no		PND 33, one day before acquisition	Morris water maze learning and memory	swim ability (control for test, straight channel)	0 E2 0 BPA	0 E2 0 BPA	no	n/a	
					no		PND 34 for 4 days		acquisition	(–) E2 (equivocal) 0 BPA	0 E2 0 low BPA (–) high BPA (not stat sig)	(M> F)	(M=F) low dose M>F high dose	BPA had no effect on acquisition in males or females . The lack of statistical significance between control males and females on days 1, 2, and 3 make it difficult to rule out chance
							PND 38		memory probe trial (retention)	0 E2 0 low BPA (–) high BPA (not stat sig)	0 E2 0 low BPA – high BPA	not stated	n/a	
Dessi-Fulgheri, 2002 Effects of perinatal exposure to bisphenol A on play behaviour of female and male uvenile rats	Sprague-Dawley dams	9 control 11 low dose 11 high dose culled to 8 pups at birth	40 μg/kg-day (low) 400 μg/kg-day (high) (0.04 and 0.4 mg/kg-day)	oral micropipette; low - 10 d before mating until weaning high - GD 14–PND 6 followed by vehicle until weaning	yes	15 pups/sex/dose control and high dose 12 pups/sex/dose low dose	PND 35, 45, 55 tests were performed between 1500 and 1900 hr under artificial dim white light, frequency of behaviors during mins 2 and 3 analyzed	Each factor was named by author and based on principal component analyses	Mean factor score for all 3 age groups pooled together was analyzed by 3-way ANOVA considering treatment, sex, age.			Based on combined frequency across all ages	Treatment X Sex P<0.1	()percentage of total variance accounted for by each factor in parentheses
								F1 - play toward males (12.9%)	nape (m), pounce (m), crawl over (m), riding (m)	0	0	not stated		
								F2 - play toward females (11.0%)	pounce (f), chase (f), nape (f), withdraw (f)	0	low + high 0	F<M (Fig. 2)	not significant	Authors claim F2 was masculinized by low BPA in females, but data not convincing
								F3 - nonsocial exploration (9.0%)	air smelling, rearing	0	0	not stated		
								F4 - defensive (8.7%)	sideways posture (m), withdraw (m)	0	0	not stated		
								F5 - low-intensity mating (7.4%)	crawl under (m or f)	low 0 high –	low 0 high –	no (Fig. 2)		Authors claim sex difference on P.406, but not supported by data.
								F6 - sociosexual exploration (7.3%)	anogenital sniffing (f), social investigation (f), self-grooming	–	low 0 high –	F<M (Fig. 3)	P<0.1 (not sig at p<0.05)	Authors claim F6 was masculinized by BPA in females, but data not convincing.
								F7 - ground exploration (7.0%)	digging, ground exploration	0	0	not stated		No data shown. Sex term significant but this doesn't mean there is a sex difference in controls.
								F8 - social interest (6.0%)	approach (m or f)	+ low – high	0 low – high	no (Fig. 4, text)		

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male	Effect of Treatment; Female	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
										+ = increase or improve – = decrease or impair 0 = no change				
Ema, 2001 Rat two-generation reproductive toxicity study of bisphenol A	Sprague Dawley rat (Crj: CD (SD) IGS) dams and adult males used for mating	25 rats/sex/group (25 litters/group)	0.2, 2, 20, 200 μg/kg-day (0.0002, 0.002, 0.02, 0.2 mg/kg-day)	gavage F0: 5-week old males and 10-week-old females for 10 and 2 weeks prior to mating period, respectively. Exposure to females continued throughout gestation and weaning F1: Selected females dosed from PND 22 for 10 weeks prior to mating. Exposure to females continued throughout gestation and lactation	no	1 pup/sex/ litter for both F1 and F2 pups	Daily beginning at PND 6	Ontogeny of behavioral developmental landmarks	surface righting	0	0	no	n.a.	Though not clearly stated, it is assumed that F1 pups selected for behavioral evaluations were same F1 pups selected for mating and continually dosed.
							Daily beginning at PND 7		negative geotaxis	0 F1; delay F2 all doses except high dose	0	no	n.a.	Authors concluded the changes were slight, not dose-dependent, and not consistent across generations.
							Daily beginning at PND 13 for mid-air righting		mid-air righting	0 F1 for all doses except earlier at 20 μg/kg 0 F2 for all doses	0 F1 for all doses except earlier at 20 μg/kg 0 F2 for all doses	no	n.a.	Authors concluded the changes were slight, not dose-dependent, and not consistent across generations. For both neg geotasix and mid-air righting reflex, magnitude of change was not increased with dose
					no	1 pup/sex/ litter for F1 pups	5–6 weeks old	3-min open field	ambulation, rearing, grooming, occurrence of urination and defecation	0	0	not reported	not reported	Based on typical 2-generation reproductive study, assume that these are same F1 animals selected for mating and were being dosed directly during this behavioral evaluation.
					no	6 F1 rats/sex/ group	6–7 weeks old	Biel multiple T-maze	elapsed time from start to touching goal ramp; number of errors	0	0	not reported	not reported	Insufficient detail
					n.a.	1 pup/sex/ litter	Not clear, but appears to be around weaning	Brain weight	F1 weanling	0	0	no	0	Sex difference not analyzed by authors, but is based on inspection of data tables
						1pup/sex/ litter	males after mating, females after weaning pups		F1 adult	0	0	n.a.	n.a.	Males were sacrificed at younger age than females so comparison of sex difference in brain weight is not valid.
						1 pup/sex/ litter	Not clear, but appears to be around weaning		F2 weanling	0	0	no	0	Sex difference not analyzed by authors, but is based on inspection of data tables.
								numerous other reproductive endpoints not reported here						

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve, – = decrease or impair, 0 = no change)	Effect of Treatment; Female	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Farabollini, 1999 (Dessi-Fulgheri lab) Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats	Sprague Dawley rat dams	9 control 11 low BPA 11 high BPA	40 μg/kg-day (low) 400 μg/kg-day (high) (0.04 and 0.4 mg/kg-day)	oral micropipette low dose - 10 days preceding conception until weaning high dose - GD 13–PND 6	no. Behaviors were videorecorded and scored with aid of software to analyze frequency and duration	control -15 low -15 high - 12 litter does not appear to be the experimental unit	85 days old at time of testing reversed-light cycle, testing during dark phase with low red and white light between 900–1900 hours	Holeboard test 5 minutes	floor with 4 holes divided into 36 squares, activity (#r of squares crossed; frequency & duration of head dipping, self-grooming, rearing, # boluses. 9 measures stat analyzed	0 low – high (frequency and duration head dipping)	– low - (duration head dipping, # crosses) – high (head dipping frequency and duration)	No	"Clear masculiniza-tion of females was not observed." Different patterns of factor loadings based on principal component analysis.	"In females, the parameters of motor activity in both tests were depressed ... as was the motivation to explore. Similarly, in males the motivation to explore was reduced. However, the most relevant finding in this sex was the reduction of anxiety, which was unmodified in the females." p. 693
								Elevated plus-maze test 5 minutes	2 open arms, 2 closed arms from a central square - entry into arm, time spent in each area; also frequency of head dips, stretched-attend posture, self-grooming, rearing. 13 measures stat analyzed.	+ low (open arm entry, % time in open arm, % open/ total entries) – high (frequency stretched-attend posture)	low - (% time in center), low + (frequency self grooming). High - (closed arm entry, % time in center, total entries)	No		
Farabollini, 2002 (Dessi-Fulgheri lab) Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats	Sprague-Dawley rat dams	7 BPA 13 control litters culled to 4 rats/sex pups cross-fostered to form 3 treatment groups each with 12 pups/sex prenatal BPA postnatal BPA control	40 μg/kg-day prenatal 40 μg/kg-daypostnatal (0.04 mg/kg-day)	oral micropipette dams treated from mating to weaning pups cross-fostered to form groups treated only prenatally or only postnatally	yes	12 pups/sex/group for prenatal-only; postnatal-only and control groups	Started at 100 days of age. Test 1 - intruder test. Test 2 one week later, sexual orientation and sexual behavior together in males, sexual activity after a second one-week interval in females. Testing at 900–1300 hr under dim light.	Intruder test: unfamiliar intruder of same sex and bodyweight introduced for 15 min	Offensive behaviors (frequency, latency) - aggressive grooming, threat, offensive sideways posture, offensive upright, chase, attack, bite, full aggressive posture.	0	0	not analyzed	n/a	Table 1 males; Table 5 females. Data was not analyzed to evaluate a sex effect on behaviors.
									Defensive behaviors (frequency, latency) - retreat, flight, crouch, defensive sideways posture, defensive upright, full submissive posture	(+)	0	not analyzed	n/a	Stat sig of questionable biological sig in %defensive/agonistic but not defensive frequency.
									Ambivalent (frequency, latency, duration) - boxing	0	0	not analyzed	n/a	
									Agonistic (total of offensive, defensive and ambivalent)	0	0	not analyzed	n/a	
								Sexual orientation test	duration of visits to male or female during 5-minute period	0	0	yes, based on inspection of data	0	In control animals, females preferred males, males had no preference (Tables 2, 6)
								Sexual activity test	males - immediately placed with stimulus female (6 variables) females - 1 week later placed in cage with experienced male but with escape hole (3 variables)	(–) slight impairment but no effect on the whole	0 (+) increase following *a posteriori* stat analyses pooling PRE and POST	n/a	n/a	Authors conclude on p. 412 that on the whole, male sexual behavior was not disrupted and there was a slight intensification of female sexual behavior. This is not consistent with hypothesis that BPA masculinizes brain.
Ishido, 2004 Bisphenol A causes hyperactivity in the rat concomitantly with impairment of tyrosine hydroxylase immunoreactivity	Wistar 5-day old rat pups	6 male pups/group (Fig 1,2)	0.02, 0.2, 2, 20 μg	intracisternal injection	automated	male pups only, n=6	4–5 weeks old	24 hr motor activity - data analyzed for 12 hr (4-5 weeks old)	spontaneous activity during 12-hour dark cycle	+ at 0.2, 2, 20 μg BPA	n/a	n/a	n/a	Only behavioral and histopathology results reported.
					not stated	not stated but assume n=6	8 weeks old	Brain Immunohisto-chemistry	tyrosine hydroxylase immunoreactivity	– (only reported for 20 μg)	n/a	n/a	n/a	No quantitative measurements. Other doses not discussed.

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve; – = decrease or impair; 0 = no change)	Effect of Treatment; Female (+ = increase or improve; – = decrease or impair; 0 = no change)	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Kawai, 2003 Aggressive behavior and serum testosterone concentration during the maturation process of male mice: The effects of fetal exposure to bisphenol A	CD-1 mouse dams	9 control 7 BPA low 7 BPA high	0, 2, 20 ng/g-day (0, 0.002, 0.02 mg/kg-day)	daily oral micropipette dose to pregnant dam GD 11–17	yes	Behavior: 4-5 male pups randomly selected males from each litter 30 control, 32 low BPA, 26 high BPA	PND 8, 12, 16	"Aggression" (authors did not really measure aggression)	Contact time during 7 minute encounter between treated 'resident' mouse and 'opponent' control mouse	+ contact time – but no attacking behavior (at 8 but not 12 and 16 wks)	n/a	n/a		There was relationship between contact time and testosterone concentration and testis weight.
						Testis weight and hormone assay: 8–14 mice/group (litter does not appear to be experimental unit)	PND 9, 13, 17 1 wk after aggression test	Biological Effects	relative testis weight	– 2 ng/kg (8, 12 wk) – 20 ng/kg (12 wk)	n/a	n/a		
									testosterone assay	0	n/a	n/a		

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve, – = decrease or impair, 0 = no change)	Effect of Treatment; Female (+ = increase or improve, – = decrease or impair, 0 = no change)	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Kubo, 2001 Exposure to bisphenol A during the fetal and suckling periods disrupts sexual differentiation of the locus coeruleus and of behavior in the rat	Wistar rat dams	5 control 5 BPA	1.5 mg/kg-day (based on estimate of water consumption of 5 mg/L water *ad libitum*)	drinking water *ad libitum* GD1–LD21	yes- activity (automated) no-rearing	n=11–14 pups per group in open field test	6 weeks of age animal placed in open-field box and measured for 10 minutes	Open field test 10 min	distance (automated)	not reported	not reported	F>M	F=M	Statistical methods very poorly reported. There were statistically significant differences in gender in controls but not in BPA-treated (i.e., the gender difference decreased after treatment). Authors claim that BPA causes demasculinization of males and defeminization of females. However this may be too strong a statement. There were no reported statistically significant differences between control and treated females or between control and treated males. Based on analyses in Kubo, 2003, Kubo would have reported these differences if he saw them. Based on inspection of graph BPA did not appear to have stat sig effect on distance moved or passive avoidance
									rearing (manual)	not reported	not reported	F>M	F=M	
									time in center (automated)	not reported	not reported	F>M	F=M	
					not stated	n=11–14 passive avoidance	7 weeks	Passive avoidance test one-trial, retention 24 hours later	latency for foot shock for entering dark compartment	not reported	not reported	M>F	F=M	
					no	n=6-7 pups per group for locus coeruleus, SDN-POA, brain weight	20 weeks	locus coeruleus	volume	not reported	not reported	F>M	F=M (authors claim M>F but not stat sig	Statistical significance in control but not in BPA-treated; case controls: F>M but in BPA M>F (but not significant).
									cell numbers	not reported	not reported	F>M	F=M (authors claim M>F but not stat sig	
					no			Sexually dimorphic nucleus of the preoptic area of the hypothalamus (SDN-POA)	volume	not reported	not reported	M>F	0	
									cell numbers	not reported	not reported	M>F	0	
					n.a.			brain weight	weight immediately after perfusion fixation	0	0	M>F	0	
					n.a.	n=12–14 for organ weights n=5–10 for hormone levels	12 weeks	reproductive organ weights and hormone serum levels	weight: testis, epididymis, prostate, ovaries, uterus. Serum levels: LH, FSH, testosterone, estradiol	0	0	+ FSH only, not tested for sig		"BPA induced no significant changes in the reproductive system."

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male	Effect of Treatment; Female	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
										+ = increase or improve – = decrease or impair 0 = no change				
Kubo, 2003 Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats	Wistar rat dams	5 control dams 6 low BPA 6 high BPA	30 μg/kg-day 300 μg/kg-day based on estimate of water consumption of 0.1 mg/L (0.03 and 0.3 mg/kg-day)	drinking water *ad libitum* GD 1–LD 21	yes, distance and duration in center no for rearing	20–24 offspring/ sex/group	6 weeks	Open field test 10 minutes	distance	0	0	F>M	F>M low dose; F=M BPA high dose	Authors report that sex differences seen in control disappeared in the BPA-high dose.
									frequency of rearing	+ BPA low 0 BPA high	0	F>M	F=M	Authors report that sex differences seen in controls disappeared in BPA high and low dose.
									duration in center	0	0	F>M	F=M	Authors report sex differences seen in the controls disappeared in BPA-high dose.
					no	7–8 offspring/ sex/group	14 weeks	locus coeruleus	volume	+ BPA low 0 BPA high	–	F>M	M>F	6.5 μg/kg/day diethylstilbestrol (DES), a synthetic estrogen with similar potency to estraidiol, had similar effects.
									cell number	+ BPA low,high	- BPA low,high	F>M	M>F	DES had similar effects.
								SDN-POA	volume	0	0	M>F	0 (M>F)	Authors report no effect of DES on SDN-POA; this could be due to lower doses.
					no	13–15 offspring/ sex/group	12 weeks (females killed at proestrus stage on the day after diestrus day)	reproductive system	organ weights, estrus cycle, serum hormone levels	0	0	n/a	n/a	Authors report effects by DES and by 1500 μg/kg/day of the phytoestrogen trans-resveratrol (RVT), but not by BPA on reproductive system.
					no	7-13 offspring/ sex/group	11–12 weeks	sexual behavior	Male behavior toward stimulus female (overiectomized, sexual receptivity hormonally induced)	- low BPA 0 high BPA	n/a	n/a	n/a	Table 4. of paper. Authors report DES had no remarkable effects.
									Female behavior toward male	0	0 BPA – RVT – DES	n/a	n/a	Table 4 of paper. Authors report DES and RVT reduced female receptivity.
Mizuo, 2004 Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice	ddY mouse dams	not reported	0.002, 0.5, 2.0 mg BPA/g diet (0.03, 75, 300 mg BPA/kg) based on assumption that adult mouse eats 150 g diet/kg bwt as per U. of Iowa Animal Care website Morphine (10 mg/kg s.c.)	diet mating to weaning	not stated	6–10 mice/group Each BPA group divided into saline and morphine group	not reported	conditioned place preference BPA-groups divided into morphine and saline group for place-preference using 1.0 mg/kg morphine previously shown not to induce place preference	900-s session time spent in each compartment BPA (B0,B0.002,B0.5,B2) rats pre-conditioned with saline compared to BPA rats pre-conditioned with METH	+ 75, 300 mg BPA/kg	n/a	n/a		Web site for mouse consumption of diet : http://research.uiowa.edu/animal/?get=mouse#Basic%20Husbandry
					tilt-cage	9–10 mice B0 and B2 group only (estimated chemical consumption is 300 mg BPA/kg)	not reported	morphine-induced increase in activity	3-hour motor activity	+ 300 mg/BPA/kg	n/a	n/a		

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male	Effect of Treatment; Female	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
										+ = increase or improve – = decrease or impair 0 = no change				
Negishi, 2003 Effects of perinatal exposure to bisphenol A on the behavior of offspring in F344 rats	F344/N rat dams	n=8 or 9 per group	4, 40, 400 mg/kg-day	Oral feeding needle (assume gavage) GD 10–PND 20	automated	All 8-9 litters/group (8 pups/litter)	PND 7, 14, 21, 28, 56, 84	body weight	weight	–	–	not stated	n/a	Weights were lower than control at younger ages, but caught up by PND 84. Dose-response was seen at PND 7 and 21 (males and females), PND 28 (m). Organ weights are not significantly different, but are so small that differences of surgical extraction could hide small biological differences.
					automated	12–27 offspring/ sex/group	4 weeks old	Spontaneous motor activity 12-hour during dark phase	total activity	0	0	not stated	n/a	
									immobile time	0	+ (40 mg/kg-day)	no	n/a	The biologic significance is questionable and appears to be spurious
					not stated	8–9 offspring/ sex/group	4 weeks old	3-day acquisition of active avoidance 50 trials/day with fixed 50-s intertria interval	2-way shuttle box, 4 week	0 (+) 40, 400 mg/kg-day	0	no	n/a	No treatment x day interaction so no effect on acquisition; + means increased avoidance which means they got better at avoiding shock.
					not stated	8–9 offspring/ sex/group	8 weeks old		2-way shuttle box, 8 week	0 (–) 4mg/kg-day; 1st day only	0	no	n/a	No treatment x day interaction so no effect on acquisition; sat 4 mg/kg-day there was a decrease in avoidance on the 1st day, but animals avoided just as well as controls by the 2nd and 3rd day.
					computer assisted, but unclear how behaviors are classified	not documented	8 weeks old	open field behavior 5 min during dark phase	grooming	+ (4 mg/kg-day)	0	no	n/a	Increase at 4 but not at 40 or 400 in males, females nonstatistically significant increase only at 4
									locomotion	0	0	not reported		
									stretching	0	0	not reported		
									rearing	0	0	not reported		

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve; – = decrease or impair; 0 = no change)	Effect of Treatment; Female (+ = increase or improve; – = decrease or impair; 0 = no change)	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Negishi, 2004 Behavioral alterations in response to fear-provoking stimuli and tranylcypromine induced by perinatal exposure to bisphenol A and nonylphenol in male rats	F344 rat dams	10–11 dams/group	0.1 mg/kg-day	oral feeding needle (assume gavage) to pregnant dams GD 3–PND 20	automatic analysis	1 male pup/litter (n=9-10) tested on all the behavioral tests No female offspring tested	8 weeks	open-field behavior 5-min during dark phase	locomotion	0	n/a	n/a	n/a	
									rearing	0	n/a	n/a	n/a	
									other	not reported	n/a	n/a	n/a	
					automated		12 weeks old	12-hr spontaneous motor activity during dark phase - counts in 2-min interval	activity	0	n/a	n/a	n/a	
									immobile time (defined as 2-min with no signal)	0	n/a	n/a	n/a	
					automated		13 weeks old	passive avoidance (1 pre-trial followed by 1 retention trial 24 hours later	retention trial latency	0	n/a	n/a	n/a	Authors report that BPA-treated groups tended to remain in light compartment longer meaning BPA improved avoidance.
									poking frequency	0	n/a	n/a	n/a	
									poking duration	0	n/a	n/a	n/a	
					not reported		14 weeks old	5-min elevated plus-maze 60 cm above floor during dark phase	open arm frequency	0	n/a	n/a	n/a	
									closed arm frequency	0	n/a	n/a	n/a	
					automated		15 weeks old	4-day acquisition of active avoidance followed by by extinction test in which no shock was given; 25 trials/day; variable intertrial interval of 10-90 sec	acquisition, latency to CS	0	n/a	n/a	n/a	This test may have been confounded by previous passive avoidance test. The authors report that BPA-treated groups tended to remain in the light compartment longer in the passive avoidance test, which means that the BPA animals learned to remain in the chamber they were placed in to avoid shock. This may have affected ability to acquire active avoidance behavior. By the 4th acquisition session, BPA animals caught up with controls.
									acquisition % correct	–	n/a	n/a	n/a	
									extinction % correct,	0	n/a	n/a	n/a	
					automatic analysis		22–24 weeks old	monoamine disruption test. Phamacologic challenge with 5 mg/kg tranylcypromine followed by 4-min open field behavior	locomotion	–	n/a	n/a	n/a	
									rearing	–	n/a	n/a	n/a	

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve, – = decrease or impair, 0 = no change)	Effect of Treatment; Female (+ = increase or improve, – = decrease or impair, 0 = no change)	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Palanza, 2002 (vom Saal laboratory) Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice Focus on in utero effects on developmental neuro endpoints	CD-1 mouse dams	F0: 9 BPA, 14 control F1: 20 oil-oil 15 oil-BPA 15 BPA-oil 15 BPA-BPA	10 μg/kg-day (0.01 mg/kg-day)	oral micropipette GD 14–18 to dams	No	Dams 20 oil-oil 15 oil-BPA 15 BPA-oil 15 BPA-BPA	Lactation Days 2–15	time spent in nested-related behavior (1x every 4 min for total of 30 observations over period of 120 min)	Incidence of nursing, licking pups, nest building, eating/drinking, grooming self, moving about cage (active), resting, forced nursing; combined groups nest-related behavior (any with pup contact) and out-of-nest behavior (no pup contact)	n/a	– BPA-oil – oil-BPA 0 BPA-BPA	n/a	n/a	The decrease in time spent in maternal-related behaviors did not result in any adverse effects in pup development.
						Litter Offspring 20 oil-oil 15 oil-BPA 15 BPA-oil 15 BPA-BPA	PND 1	Day 1 measurements	# pups per litter, sex-ratio, body weight of each pup. Litters culled to 10, 5 each males and females when possible.	0	0	no	n/a	
						8 litters/group (all 10 pups/litter evaluated)	PND 3, 5, 7, 9 (also 13 for body weight)	Offspring's postnatal development	cliff drop aversion	0	0	no	n/a	Time taken for pup to turn away from cliff until parallel to edge of table. Animals falling asleep or that fell off assigned maximum latency of 120 sec.
									righting reflex	0	0	no	n/a	BPA-oil had slightly longer delay at PND 3 that was not statistically significant. Authors conclude on p. 59 that there is no disruption in growth or reflex measures of offspring of BPA-oil group.
									body weight	0	0	no		
Sashihara, 2001 Effects of central administration of bisphenol A on behaviors and growth in chicks	4 day old male chicks, Julia type	13 control 12 low 12 high	100, 200 μg BPA central injection	intracerebral injection	No video recording and audio analysis every second for 5 minutes for behaviors	organ weight: 10 control, 8 low, 9 high body weight: 13 control, 12 low, 12 high	organ weight: 20 day old body weight (4, 5, 6 day old)	body and organ weight	4 organs - liver, kidney, testis, brain n=10 - control, 8 - 100 μg, 9 - 200 μg	0	n/a	n/a	n/a	No effect noted on food intake, total body weight, or organ weight. Methods section was very unclear.
						7 chicks/treatment	12? day old (Results p. 278: 8 days after injection)	5-min following isolation (distressed animals)	jumping, distress vocalization, locomotor activity, duration of crouching	0 movement + distress vocalization	n/a	n/a	n/a	Distress vocalization gives linear dose/response increase, others 0; subjective evaluation of vocalization.

Summary of BPA studies (cont.)

First Author, Year Title	Animal Dosed	BPA Treatment Groups (animals dosed with BPA)	BPA Doses	BPA Exposure Route and Duration	Blind Evaluation? (assume "no" if not mentioned)	Group Size for Endpoint	Age (age animals were tested on endpoint listed at right)	Endpoint (focus on neurobehavior, others reported if considered relevant)	Specific Measurement	Effect of Treatment; Male (+ = increase or improve; – = decrease or impair; 0 = no change)	Effect of Treatment; Female (+ = increase or improve; – = decrease or impair; 0 = no change)	Sex Difference in Controls	BPA Effect on Sexual Differences	Comments
Sato, 2001 The effects of prenatal exposure to ethinyl estradiol and bisphenol-A on the developing brain, reproductive organ and behaviour of mouse	Jcl-ICR mouse dams	28 dams/group	100 mg/kg-day BPA; 0.2 mg/kg-day ethinyl estradiol (EE) no live births 0.02 mg/kg-day EE (EE reported in comments column; in general. EE did not have effects similar to that of BPA)	subcutaneous injection to dams GD 11–19	No	38 control 17 EE 51 BPA	as appropriate for developmental endpoint	sensorimotor development	pivoting, righting, straight-line walking, grasp reflex	0	0	not measured	n/a	Righting reflex: EE<BPA; EE=control Straight-line: EE<control, BPA
						56 control 37 EE 62 BPA	PND 60	3-min openfield activity	grooming	+	+	not measured	n/a	EE>control,BPA
									rearing	0	0	not measured	n/a	EE>BPA
									line-crossing, inner and outer fields	0	0	not measured	n/a	Inner: EE>control Outer: EE>BPA
									defecation	–	–	not measured	n/a	EE>control
									latency	0	0	not measured	n/a	EE=control=BPA
						PND 40: 30 control, 19 EE, 12 BPA; PND 60: 51 control, 68 EE, 52 BPA	PND 40 and 60	diameter of tractus mamillothalamics	PND 40	0	0	not measured	n/a	EE<BPA EE=control
									PND 60	0	0	not measured	n/a	EE<BPA; EE=control
						PND 40: 15 control, 15 EE, 20 BPA; PND 60: 15 control, 68 EE, 52 BPA	PND 60	male reproductive organs	diameter of seminiferous tubules	–	n/a	n/a	n/a	EE < control
									number of cell layer	–	n/a	n/a	n/a	EE<control
Suzuki, 2003 Prenatal and neonatal exposure to bisphenol A enhances the central dopamine D1 receptor-mediated action in mice: enhancement of the methamphetamine-induced abuse state	Adult ddY mouse dam	not reported	0.002, 0.5, 2 mg BPA/g diet (0.03, 75, 300 mg /kg-day) based on assumption that adult mouse eats 150 g diet/kg bwt (http://research.uiowa.edu/animal/?get=mouse#Basic%20Husbandry) Methamphet-mine s.c. range 0.125–2 mg/kg	diet mating to weaning	not stated	6–10 mice/group Each BPA group divided into saline and METH group	not reported	conditioned place preference BPA-groups divided into METH and Saline group for place-preference using 0.5 METH previously shown not to induce place preference	900-s session time spent in each compartment BPA (B0, B0.002, B0.5, B2) rats pre-conditioned with saline compared to BPA rats pre-conditioned with METH	+ 0.03, 75, 300 mg BPA/kg	n/a	n/a		METH demonstrated to cause place preference in untreated animals. 0.5 METH is NOAEL for place preference BPA results in 0.5 METH place preference in dose-related manner. B2 effects can be blocked by D1-antagonist.
					ambulameter (tilt-cage)	9–10 mice B0 and B2 group only (estimated chemical consumption is 300 mg BPA/kg)	not reported	methamphet-amine (METH) induced increase in activity	3-hour motor activity	+	n/a	n/a		BPA potentiates METH increase in locomotion.
							not reported	sensitization	10-min motor activity sensitization to METH induced increase in activity	+	n/a	n/a		Hyperlocomotion was increased in 2.0 mg BPA-treated animals (2.0 was only level tested)

Appendix 1

Summary of Individual Papers

Brief Summaries of Published Papers Evaluating Neurobehavioral Effects of Bisphenol A (BPA)

The following summaries are intended to be used in conjunction with the BPA Neurobehavioral Studies table. The upper right-hand corner of each summary identifies the first author and year that the paper was published for easy cross-reference to the BPA table. These summaries are notes of Dr. Abby Li's evaluation of some of the strengths and weaknesses of the papers.

Some papers that contained neurobehavioral data also reported data on sexual behaviors, reproductive endpoints, neuropathology, and neurochemistry. The focus of this evaluation is on the neurobehavioral data.

Two papers (Ishido et al. 2004, Sashihara et al. 2001) that were mentioned briefly in this report are also summarized at the request of APC. These papers are outside the initial scope of this overall review, which focused primarily on behavioral effects of exposure to BPA using routes of exposure other than direct injections into the brain.

Adriani, W., W. Della Seta, F. Dessi-Fulgheri, F. Farabollini, and G. Laviola. 2003. Altered profiles of spontaneous novelty seeking, impulsive behavior, and response to D-amphetamine in rats perinatally exposed to bisphenol A. Environ. Health Perspect. 111(4):395–401.

Strengths

Complex behaviors requiring training of animals were evaluated. The behavioral tests appear to have been well-conducted. Time of testing was balanced across groups for the novelty preference test. The observer was blind for the amphetamine test. There were clear criteria for evaluating behavior (e.g., number of line crossings with both forepaws scored).

The authors statistically analyzed the results, with litter as the experimental unit. The litter size of nine, with one male and one female tested per litter, is adequate, although in developmental guideline studies, the requirement is typically 16–20 litters/group.

Weaknesses

Errors in data reporting: The final published paper has several errors in the figures that make the published paper difficult to follow. These errors did not occur in the on-line version of this paper. Assuming that the text is correct in the published paper, there was mislabeling of control and treated groups in Figures 1C, 1D, and 2, as well as an error in labeling of the y-axis in Figure 3B.

Authors interpreted rat behavior in terms of human behaviors in a manner that does not appear to be objective: One illustration of this is the authors' claim that BPA caused increased activity in a novel chamber, which may be an indication of novelty-induced stress. It is just as plausible to conclude from the presented data that increased activity in a novel environment reflects increased motivation to explore a novel environment, which is an adaptive response. The authors tend to anthropomorphize the rat behavior in this study. Attributing human behaviors to the rats hampers the objectivity of the authors' interpretations of study data.

Authors interpreted changes in behavior as evidence of effect on ontogenesis of the central neurochemical system, but did not demonstrate neuropharmacologic effects: The authors frequently made strong statements that the behavioral changes are indicative of ontogenic neurochemical effects. The logic used to support this statement is that BPA caused behavioral effects, and neurotransmitter changes have been reported by other investigators to cause similar general behavioral effects. Therefore, BPA caused ontogenic effects on the neurochemical system. This logic suggests that if A then C AND if B then C, THEN A causes B. This association of causality does not stand up to critical scrutiny. These conclusions are not supported by the data and should be viewed cautiously as a hypothesis, because a) the authors did not conduct any mechanistic studies directly relating changes in behavior to altered endocrine status or neurotransmitter levels, and b) the authors did not test known estrogenic chemicals (e.g., ethinyl estradiol) or neuropharmacologic agents on the behavioral endpoints to compare responses with those observed in BPA-treated animals.

Authors used the term "feminization" loosely and inappropriately: Terms like "feminization" imply an impact on the sexual differentiation of the brain. The authors interpreted the reduction in inadequate responding on the operant behavior as demasculinization or feminization because the frequency of inadequate responding in BPA-treated males was reduced to levels similar to that of control females. However, the authors provided no data that related *in utero* effects of BPA to sexual differentiation of the brain. It should be noted that a reduction of inadequate responding could be regarded as an improvement in operant behavior. Of even more significance is that the authors reported that BPA reduced impulsivity in both sexes based on the key operant measurement of the number of nose pokes associated with delayed but larger reinforcement.

Unknowns

Activity monitoring in amphetamine test may not be sufficiently sensitive to make a conclusion about BPA effects: Based on the data in Figure 4, the increased activity at 1 mg/kg amphetamine was not statistically significant in control females. This indicates that the method of evaluation may not have been sufficiently sensitive to detect increases in expected activity

with a 1-mg/kg dose of amphetamine in control males and females. It is not clear whether this test was balanced over time with respect to treatment (as was indicated for other tests). To be useful, this test would need to be repeated, preferably with exposure levels that show a dose-response for amphetamine. The authors also did not report effects on grooming.

Overall Assessment

BPA did not have a consistent adverse effect on several behaviors. BPA did not appear to have an effect on activity level (Figure 4, compare oil and BPA groups that were treated with saline). BPA may have improved performance (decreased impulsivity) of a complex operant behavior designed to evaluate effects on impulsivity. BPA reduced time spent in a novel environment in females but not in males, but increased activity level in the novel environment in both sexes.

Aloisi, A.M., D.D. Seta, C. Rendo, I. Ceccarelli, A. Scaramuzzino, and F. Farabollini. 2002. Exposure to the estrogenic pollutant bisphenol A affects pain behavior induced by subcutaneous formalin injection in male and female rats. Brain Res. 937:1–7.

Strengths

Data were presented clearly in tables or graphs. Measurements of estradiol, testosterone, and corticosterone were made. A cross-fostering experimental design was used so that effects on pups exposed prenatally could be compared with effects on pups exposed postnatally.

Weaknesses

There was no mention of whether observers were unaware of treatment. The individual pup and not the litter was the experimental unit. Presumably, the formalin test caused pain and increased licking, flexing, and jerking. The nociceptive measure depended on subjective evaluation of licking, jerking, etc. BPA increased licking and flexing but reduced jerking, which is not a consistent pattern of effect. The authors explained this by suggesting that there is a different anatomic basis for licking and flexing compared to jerking, but the supportive evidence was weak. Authors acknowledged that BPA had no statistically significant effect on locomotion or exploration in formalin or sham treated animals. However, they claimed that BPA had opposite modifications in males and females with locomotor and exploratory activity. The data did not support this conclusion in a consistent manner. Although there was a tendency for BPA to decrease activity in "sham" males, the activity measurements were too variable to make any conclusions about patterns of effects in males compared to females. The authors stated in the abstract that "BPA modified the activity of neural pathways and/or centers involved in nociception and pain in a sex-related and exposure-related manner." This conclusion was not supported by data.

Unknowns

The validity of the formalin test as a measure of pain behavior cannot be determined based on this paper alone. This paper should also be evaluated within the context of a previously cited paper by this author on this behavioral test. The authors performed multiple statistical

comparisons for individual time periods (e.g., first 5 minutes for licking, 15–40 minutes, 40–60 minutes). This selective analysis may be justified based on earlier experiments.

Overall Assessment

The only conclusion that can be made from this study is that BPA may affect pain behavior as measured by the formalin test. However, the increase in flexing and licking does not appear to be consistent with the decrease in paw-jerk durations.

Carr, R.L., F.R. Bertasi, A.M. Betancourt, S.D. Bowers, B.S. Gandy, P.L. Ryan, and S.T. Willard. 2003. Effects of neonatal rat bisphenol A exposure on performance in the Morris water maze. J. Toxicol. Environ. Health Part A 66:2077–2088.

Strengths

Behavioral test is a very well-established method: Morris water maze has been used extensively to study the neurobiological mechanisms that underlie spatial learning and memory, age-associated changes in spatial navigation, and the ability of psychopharmacological agents, lesions, or gene mutations to influence specific cognitive processes. The authors also used a well-accepted experimental design, which is sometimes termed "place navigation task." In this task, the place of the platform was kept the same each day, but the starting point of the rat varied. This method evaluates long-term spatial memory and learning. It is a difficult task because the platform no longer can be found using a single visual cue. Instead, the animal must construct configural associations to solve the task.

Swimming ability was evaluated: The authors conducted a straight-channel swim test so animals could become accustomed to swimming in the water, and to control for possible confounding effects on swimming ability.

Effect of estrogenic chemical on laboratory procedures was included in study: The authors tested the effects of 17β-estradiol (E_2) on their specific experimental procedure. This is an important internal positive control for possible estrogenic effects on the behaviors measured that can help generate hypotheses about whether or not BPA causes estrogenic-like effects. E_2 had no effects on females and very slight effects on increasing acquisition time in males during the third of four trial days. Ultimately, E_2 had no adverse effects as measured in this test.

Estrogenic exposure controlled for in diet: Rats were placed on casein-based rodent chow to eliminate natural phytoestrogens from the diet.

Weaknesses

Number of litters/treatment group was not reported: Authors stated that 10 male and female rat pups were assigned to each treatment group, but did not specify the number of litters represented. The litter is the appropriate experimental unit.

Control of important confounding variables was not reported: It was not reported whether the time of testing was balanced across treatment groups.

Control female acquisition: On the final acquisition trial day, the latency to find the platform was approximately 20–30 seconds in control females compared to 12–22 seconds in control males. This latency appears to be long. If the rats have not learned where the platform is, then it is more difficult to interpret effects on performance during the probe test.

Sex difference in acquisition needs to be evaluated cautiously: The difference between the behavior of males and females was not robust enough to convincingly support the authors' conclusion that treatment with low BPA or E_2 eliminated a sexual difference in behavior. Reporting of the statistical analysis (ANOVAs and posthoc tests) was not complete.

Overall Assessment

The authors claim that 100 μg/kg of BPA (and not 250 μg/kg) eliminates normal sex difference in acquisition of a spatial task. Yet, there were no treatment-related effects of BPA on acquisition in either female or male rats. There was very little difference in magnitude in the two dose levels so it's misleading to consider these as low and high dose levels. This is largely a negative study with possible effects on retention of performance in the Morris water maze in high-dose females. These effects cannot be related to estrogenic effects, because E_2 had no effect on overall acquisition or retention in males or females.

Dessi-Fulgheri, F., S. Porrini, and F. Farabollini. 2002. Effects of perinatal exposure to bisphenol A on play behaviour of female and male juvenile rats. Environ. Health Perspect. 110(3):403–407.

Strengths

The observers were blind to treatment level. There was a predefined list of social and nonsocial behaviors that had explicit operational definitions. Methods used were clearly described. The principal component method was used to detect relationships between different behavioral endpoints. This is a useful statistical tool that allows investigators to reduce the number of variables and to detect structure in the relationships between variables—that is, to classify variables.

Weaknesses

The analysis was based on frequency of behaviors displayed during a relatively short period of time (between the second and third minutes). This may not be long enough to be representative of the social behaviors measured. The authors' conclusions were dependent on the validity of the principal component analyses to group different behaviors into factors. These factors were further analyzed by pooling the three age groups and performing statistical comparisons on mean factor scores. The authors considered any BPA change in either direction as either masculinization or feminization, which is inappropriate. In addition, the authors defined the statistically derived factors as "social interest," "sociosexual exploration," "ground exploration," "defensive behavior." These terms are completely subjective and present a danger of misleading the reader that these behaviors are directly relevant to humans. The authors' conclusions that BPA caused masculinization of female behaviors in "play" and "sociosexual exploration" depended on multiple layers of statistical analyses and questionable assumptions. In fact, even if one accepts the authors' analyses, the treatment × sex interaction term from the ANOVA was not statistically significant for "play" and $0.05<p<0.1$ for "sociosexual exploration." To test the generalizability of the findings from principal component analyses, a second research study needs to be conducted to see whether the findings can be verified. Alternatively, some researchers split the sample randomly into two halves, conducting principal

component analysis on each half and comparing the results. The authors have not validated the findings of the principal component analyses. There was no treatment × sex × age effect for all eight factors. Only one factor was significant for treatment × sex, but $p>0.05$.

Unknown

The results of this study depend completely on the statistical and biological validity of the principal component analysis, including (a) whether the criteria used to group behaviors is statistically valid (some correlation coefficients appeared to be low); (b) to what extent did the investigators preliminarily select measures to be entered into the analyses as they did in previous papers (Farabollini et al. 1999); and (c) whether further analyses of principal component analysis factors by ANOVA are appropriate (including pooling together factors from three age groups). This paper should be analyzed by a statistician.

Overall Assessment

This paper has been quoted by other researchers studying BPA as evidence that BPA affects social behaviors. The apparent effect of BPA on "social" and "play" behavior depends on multiple layers of statistical assumptions (e.g., ANOVA of pooled factors from principal component analyses). The authors used terms such as "sociosexual exploration," "social interest," and "nonsocial exploration," which imply relevance to human behaviors that has not been validated. The authors tended to overstate the biological significance of their analyses as evidence of masculinization of female behavior, ignoring the largely negative statistical significance of treatment × sex and treatment × sex × age in behaviors. The conclusions from this study should be regarded as generating hypotheses that require further testing.

Ema, M., S. Fujii, M. Furukawa, M. Kiguchi, T. Ikka, and A. Harazono. 2001. Rat two-generation reproductive toxicity study of bisphenol A. Reprod. Toxicol. 15:505–523.

Strengths

This was a complete two-generation reproduction study using 25 litters/dose group. Open-field observations and ontogeny of behavior were evaluated using large sample size. The litter was the statistical unit of analysis. Reproductive and developmental endpoints were also measured.

Weaknesses

The criteria used to measure rearing, grooming, and ambulation during 3-minute open-field evaluation are not described. The methods for and results of the water-filled multiple T-maze were not adequately described. The authors indicated that statistically significant changes in functional development, such as completion of mid-air righting reflex and negative geotaxis reflex, were found. The authors stated that the changes were slight and were neither dose-dependent nor consistent across generations. I agree with this conclusion. However, historical control data from the laboratory would have strengthened these statements.

Overall Assessment

The authors concluded that BPA had no effect on functional development.

Farabollini, F., S. Porrini, and F. Dessi-Fulgheri. 1999. Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats. Pharmacol. Biochem. Behav. 64(4):687–694.

Strengths

Operationally defined behaviors were used. Observers were unaware of treatment group. Results and methods were reported clearly. Principal component method was used to determine whether certain behaviors could be correlated with each other. The principal component method is a useful tool that allows investigators to reduce the number of variables and to detect structure in the relationships between variables that may not be obvious. The appropriate use of this method includes conducting a separate experiment to validate the factor loadings.

Weaknesses

Authors conducted post-hoc multiple comparisons even when two-way ANOVA was not statistically significant and involved closely related variables (e.g., both frequency and duration of behaviors were analyzed for many of the behaviors). In addition, multiple comparisons of percentage of measurements were made. Authors ascribed human behaviors to the rat behaviors. For example, more frequent entry into an open arm of the plus maze and reduction in stretched-attend behavior was considered evidence of reduced anxiety. Small increases in self-grooming were interpreted as a form of displacement of conflict situations. There is no evidence that any of these behaviors actually reflect how the rat "feels." The authors used a circular argument by using the results of the principal component analyses to identify behaviors as measures of "anxiety" or "motivation to explore." They do this by evaluating the results of the principal component analyses, which relates different behaviors into factors. For example, if the factor analyses linked different behaviors to a behavior (e.g., head dips are related to stretched-attend) that they already pre-defined as a measure of anxiety or motivation to explore; then they concluded that the behavior (e.g., frequency of head dips on holeboard test) must also be related to "anxiety." There is no evidence that any of the statistically significant effects have any functional significance or can be related to how the animal feels (e.g., anxious, motivated to explore). The principal component analysis is a useful tool to help identify relationships between different variables. It should be considered as hypothesis-generating. The conclusions

cannot be regarded as valid until the hypothesis is tested by a separate experiment to determine if the same overall patterns of factor loadings can be found.

Unknown

The differences in magnitude in some measures appear to be small, such as numbers of entries into various sections (2.5 vs. 0.9; 2.5 vs. 4.2), number of stretched-attend postures (2.6 vs. 4.6), and frequency of head-dipping (2.4 vs. 5.8). The biological significance of these small changes is uncertain and needs to be evaluated relative to the laboratory's historical control levels and available positive control data. It is unknown whether the principal component analysis was influenced by the authors' preliminary selection of measures "to avoid redundance."
A statistician should evaluate this paper.

Overall Assessment

This is a largely negative study, considering the multiple comparisons made on different behavioral endpoints, some of which are closely related (frequency and duration of same behavior, or ratios of behavioral endpoints). For example, in low-dose males, increase in open arm entries, percent time in open arms, percent open/total entries, and a decrease in stretched-attend posture were all statistically significant. Three of these behaviors are really the same measure evaluated in different ways. Based on principal component analyses and the authors' assumptions regarding the biological significance of the behaviors, the authors concluded that BPA decreased parameters related to motor activity in females, and "motivation to explore" in males and females. They concluded that BPA appeared, in males but not in females, to reduce behaviors that the authors associate with anxiety. The authors also concluded that, contrary to their expectation based on the estrogenic action of BPA and the critical period of administration, a clear masculinization of females was not observed. The authors concluded that there were no substantial differences in effects of prolonged treatment with low dose and shorter exposure to high dose. They suggested that their results raise concern for public health, given that there is prolonged human exposure to low concentrations. This overstates the biological significance of the results. The authors did not provide evidence that the statistically significant behaviors can

be considered adverse effects. In fact, based on the authors' analyses, BPA reduced "anxiety," which could be considered a beneficial effect.

Farabollini, F., S. Porrini, D.D. Seta, F. Bianchi, and F. Dessi-Fulgheri. 2002. Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. Environ. Health Perspect. 110(Supplement 3):409–413.

Strengths

The observers were unaware of treatment level and used operational definitions to describe the behavioral observations.

Weaknesses

Although operationally defined behaviors were used, it is unclear how the specific subjective behaviors were evaluated. For example, the authors did not explain the criteria used to distinguish between aggressive and normal grooming or between offensive and defensive sideways posture. Authors tended to overstate biological significance of the very few statistically significant effects and attributed human behaviors to the rat behaviors (e.g., anxiety, motivation to explore). Authors conducted multiple comparisons of the different behaviors as well as of ratios and percentage of behaviors. No correction was made for these multiple comparisons. Authors conducted statistical analyses *a posteriori* on sexual behavior of females following combination of data from both the prenatal group and postnatal group.

Overall Assessment

This paper is largely a negative study. There were no statistically significant effects on any of the directly measured behaviors in the intruder test. The only statistically significant effect noted was a slight increase in defensive behavior when expressed as ratio of defensive behavior to total agonistic behavior. There was no effect of BPA on sexual orientation. The authors concluded that, on the whole, male sexual behavior was not disrupted, and there was a slight intensification of female sexual behavior. They stated that these effects are not consistent with the hypothesis that BPA masculinizes the brain. The authors concluded that even slight changes in the sphere of sexual behavior may have important consequences in terms of fitness and welfare at the individual level, with consequences on population dynamics.

Ishido, M., Y. Masuo, M. Kunimoto, S. Oka, and M. Morita. 2004. Bisphenol A causes hyperactivity in the rat concomitantly with impairment of tyrosine hydroxylase immunoreactivity. J. Neurosci. Res. 76:423–433.

Strength

Motor activity was measured for 24 hours by automated system.

Weakness

Intracisternal injection is not a relevant route of human exposure. The methods section did not provide sufficient detail on exactly how and where the site of injection was determined.

Overall Assessment

Intracisternal injections of BPA increased activity during 12-hour dark cycle and decreased tyrosine hydroxylase immunoreactivity in the substantia nigra. The route of exposure is not relevant for human health risk assessment.

Kawai, K., T. Nozaki, H. Nishikata, S. Aou, M. Takii, and C. Kubo. 2003. Aggressive behavior and serum testosterone concentration during the maturation process of male mice: The effects of fetal exposure to bisphenol A. Environ. Health. Perspect. 111(2):175–178.

Strengths

Observers were unaware of treatment level. Testosterone levels and testes weight were measured in this study. Correlation between contact time and testosterone concentration was statistically analyzed.

Weaknesses

The authors concluded that male mice exposed to bisphenol A were more aggressive and had a reduction in relative testis weight, compared with controls, at 8 weeks of age. Aggression was measured as cumulative time that the test mouse had body contact with opponent, including sniffing or attacking. The authors reported that no mice showed any indication of attacking behavior. There was insufficient evidence that the cumulative time of contact is a reliable measure of aggression. Contact time included non-aggressive sniffing behavior. It would be more accurate to state that there was an increase in contact time during which mice sniffed each other. The litter was not the experimental unit. Testis weight and hormone levels were based on 8–14 mice, representing an unknown number of litters.

Overall Assessment

The authors concluded that the high- and low-dose BPA groups showed increased "aggressive behavior," yet the low-dose group actually had a smaller relative testis weight than did controls, and there were no effects on testosterone level. There was no correlation between the "aggressive behavior" and relative testis weight and testosterone level. The investigators did not provide evidence that increased contact time in a 7-minute period is a valid measure of "aggression," especially when none of the mice demonstrated any attacking behavior. This study does not provide evidence that BPA causes adverse neurobehavioral effects in mice.

Kubo, K., O. Arai, R. Ogata, M. Omura, T. Hori, and S. Aou. 2001. Exposure to bisphenol A during the fetal and suckling periods disrupts sexual differentiation of the locus coeruleus and of behavior in the rat. Neurosci. Lett. 304:73–76.

Strengths

Brain and hormone levels were measured in the same study as behavioral effects.

Weaknesses

There were only five litters/group, and the litter was not considered the experimental unit. There was inadequate discussion of how animals were selected for the different endpoints with respect to representing different litters. The description of behavioral methods was not complete (e.g., box placed in center of open field). There was inadequate discussion of the statistical analyses conducted. Rearing, cell volume, and cell density measurement were not conducted blind to treatment level. Single-trial passive avoidance behavior as a test for cognition can be confounded by motor activity, because higher activity can result in decreased latency to cross to the dark side where the shock is. Therefore, these results need to be repeated using adequate sample size before any definitive conclusions can be made.

Unknown

The authors reported a difference between sexes in BPA groups compared to controls for motor activity and passive avoidance. An equally important observation is whether BPA had effects on males or females when compared against their respective controls. It is unknown whether the authors conducted this statistical comparison, although it would be surprising if they did not.

Overall Assessment

BPA consistently decreased the sex difference in four closely related behaviors. Horizontal movement, rearing, duration in center area, and one-trial passive avoidance can be considered different measures of activity. BPA also reduced the sex difference in morphometric measurements of the locus coeruleus but had no effect on the sexually dimorphic nucleus of the

preoptic area in the hypothalamus. It is not clear whether the absence of reported statistical significance in BPA males and females compared to their respective controls indicates that there were no effects, or simply did not conduct this statistical analysis. These results should be considered preliminary findings that need to be repeated in a larger study, with the litter as the experimental unit and with the observers who measure the brain areas unaware of treatment level. Historical control data are of special importance, because the differences in size and volume between sexes and after BPA treatment were relatively small.

Kubo, K., O. Arai, M. Omura, R. Watanate, R. Ogata, and S. Aou. 2003. Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci. Res. 45(3):345–356.

Strengths

The methods section was much clearer and provided more detail than Kubo et al. (2001). The sexual behaviors were analyzed in terms of patterns of consistent effects. Two other estrogenic chemicals were also evaluated. One is *trans*-resveratrol (RVT), which is a phytoestrogen found in grapes, red wine, peanuts and other fruits. The other is diethylstilbestrol (DES), a synthetic estrogen. The results of this paper were similar to those found at a higher dose in Kubo et al. (2001). (The scope of my review was to evaluate neurobehavioral data. A separate evaluation of this paper should be conducted within the context of the literature on BPA's effect on sexual behavioral and reproductive measurements and the literature on DES effects on sexually dimorphic nuclei.)

Weaknesses

The individual pup, not the litter, was considered as the statistical unit of analysis for the behavioral and neuropathology endpoints. There were no corrections for multiple comparisons. It was unclear how animals were selected for different tests and whether they were selected to represent different litters. The morphometric measurements were not conducted blind.

Unknown

Contrary to expectations, DES had no effect on the sexually dimorphic nucleus of the preoptic area which is known to be responsive to estrogen during development. The authors hypothesize that this was due to lower doses than were used in other studies in which effects were noted. The range of the historical control values from this laboratory would aid in interpreting the results for both the morphometric and behavioral measures. The following table estimates activity levels for control males in an open-field test with a center box for 10 minutes. There appears to be significant variability between two different control groups.

Group	Distance (m)	# Rears	Staying in Center(s)
Kubo (2003) control males	21	16	50
Kubo (2001) control males	12	10	10

Overall Assessment

BPA decreased difference in sexes in motor activity and in the size of the locus coeruleus. These effects are consistent with results from a study conducted previously at higher doses by the same authors. In addition, the authors included DES as a positive control. DES had effects on the locus coeruleus similar to those of BPA. However, DES had no effect on SDN-POA, which is a somewhat unexpected finding that needs to be evaluated within the context of the larger literature. Changes in the size of nuclei could be considered adverse effects. Historical control data would be especially useful in understanding the significance of these relatively small changes. The limitation of this study is that a small sample size of five dams per dose group was used. However, the authors are repeating an effect that was reported previously at higher dose levels (Kubo et al. 2001).

Negishi, T., K. Kawasaki, A. Takatori, Y. Ishii, S. Kyuwa, Y. Kuroda, and Y. Yoshikawa. 2003. Effects of perinatal exposure to bisphenol A on the behavior of offspring in F344 rats. Environ. Toxicol. Pharmacol. 14:99–108.

Strengths

Litter size was eight or nine per treatment.

The behavioral tests used were described well.

Weaknesses

Observers were aware of treatment group. There were no clear criteria established to define "grooming," "stretching," and "other" behaviors. Although fairly well-established tests were used, the data-evaluation focused on measurements of uncertain biologic significance. (See discussion of results for specific examples.)

Eight or nine litters were tested, but it was not clear how pups were selected for behavioral observations (e.g., 12–27 pups/group were tested on activity; 9–18 per group were evaluated on open-field behavior). The litter did not appear to be the statistical unit of analysis.

Authors reported no significant interactions between treatment × day based on the repeated measures ANOVA at both 4 and 8 weeks of age. However, post-hoc ANOVAs appeared to have been conducted for each day. For example, the results section indicated that there was a decrease in avoidance at 8 weeks at 4 mg/kg, but it was not until later in the discussion section that it was clarified that the statistical significance occurred only on the first day.

Avoidance: BPA had no effect on this learning ability, except at 4 weeks. Males treated with 40 and 400 mg/kg-day BPA increased avoidance of the shock, which is an adaptive (beneficial) effect rather than an adverse effect. The general lack of effect on avoidance rate, plus the absence of treatment × day interaction, indicates that the initial decrease during the first day had no functional consequence on acquisition of the avoidance behavior. However, the authors interpreted this initial decrease in avoidance as a "reduced motivation to escape from fearful condition." This test was not designed to measure "reduced motivation."

Overall Assessment

This study demonstrated that BPA had no effect on acquisition of avoidance behavior or on spontaneous motor activity. The few effects noted are of questionable biological significance and did not occur in any pattern that suggests adverse effects.

Negishi, T., K. Kawasaki, S. Suzuki, H. Maeda, Y. Ishii, S. Kyuwa, Y. Kuroda, and Y. Yoshikawa. 2004. Behavioral alterations in response to fear-provoking stimuli and tranylcypromine induced by perinatal exposure to bisphenol A and nonylphenol in male rats. Environ. Health Perspect. 112(11):1159-1164.

Strengths

This paper provided clear descriptions of methods and results. The litter was the experimental unit of analysis, in which one male pup/litter (10 litters) was tested on a series of behavioral tests, including an open-field test, a measurement of spontaneous activity during a dark phase, a step-through passive avoidance test, an elevated plus-maze, and a two-way shuttle box avoidance test. In addition, a pharmacologic challenge test was evaluated in which behavioral responses to tranylcypromine (Tcy), a monoamine oxidase inhibitor, were investigated. Tcy is a non-selective monoamine oxidase inhibitor that increases serotonin, dopamine, and norepinephrine both centrally and peripherally.

Weaknesses

The authors suggested that increased latency for active avoidance on the first session of trials is an indication of being more sensitive to fear-inducing shock and that perinatal BPA exposure may render male offspring exceedingly vulnerable to intolerable levels of fear. This hypothesis is based on anecdotal anthropomorphic reporting of behavior during the test that was not a systematic planned evaluation. Another possibility is that the passive avoidance test may have interfered with active avoidance testing in the same animals. In the passive avoidance test, animals avoided shock by remaining in the lighted chamber they were originally placed in. In the active avoidance test, animals avoided shock by moving to the opposite chamber.

Overall Assessment

This study stands out among the behavioral studies for BPA, because the sample size was 9–10 rats/group, with each rat representing a different litter. Many of the BPA studies evaluating neurobehavioral endpoints did not consider the litter as the experimental unit and had very few litters represented. Perinatal BPA exposure had no effects on several behaviors,

including open-field activity and rearing, 12-hour motor activity, passive avoidance test, and frequency of entries into open arms and closed arms of an elevated plus-maze. However, perinatal exposure to BPA appeared to reduce acquisition of active avoidance behavior, although rats performed just as well as control rats on the last trial day. This result needs to be repeated in animals that are not evaluated using the passive avoidance test. BPA attenuated the effects of Tcy to increase activity. This effect also needs to be repeated using a dose-response to Tcy, because motor activity can be variable.

Palanza, P., K.L. Howdeshell, S. Parmigiani, F.S. vom Saal. 2002. Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ. Health Perspec. 110(S3):415-422.

Strengths

The litter was the experimental unit, with 15–20 dams per treatment group. All pups in the litter were evaluated for body weight, development of cliff avoidance, and righting reflex. The behaviors were operationally defined. Animals were frequently evaluated over a 120-minute period of time during the dark phase.

Weaknesses

The authors did not state whether maternal behaviors were conducted blind to treatment level, or whether the time of testing was balanced across treatment level. Many different normal maternal behaviors were measured. Statistically significant differences in time spent in these normal behaviors were assumed to be adverse and were not discussed in relation to whether these changes have biologically meaningful effects on function. For example, a decrease in time spent nursing was not related to whether pups had milk in their stomachs and increases in body weight. A small increase in time spent building a nest is not necessarily better maternal behavior if the dam has established a nest for the litter already. It should be noted that although all the behaviors were normalized to the same unit, the scale for the y-axis is very different for each endpoint, which emphasizes relatively small differences for many of the behaviors.

Unknown

There can be a wide range of normal maternal activity in and outside of the nest. There are no historical control data available that would help determine whether the relatively small changes are within normal control behavior.

Overall Assessment

In the BPA-oil and oil-BPA treated dams, there were decreases in percent time spent on nesting-related behaviors, but no effects were seen in the BPA-BPA group. The effects on maternal behavior had no impact on pup development as measured by growth, cliff avoidance, and righting reflex. Thus, the statistically significant effects had no functional consequences on the developing pup and should not be considered adverse effects.

Sashihara, K., A. Ohgushi, R. Ando, T. Yamashita, T. Takagi, T. Nakanishi, T. Yoshimatsu, and M. Furuse. 2001. Effects of central administration of bisphenol A on behaviors and growth in chicks. J. Poult. Sci. 38(4):275–281.

Strengths

Intracerebral injection into the brain is one way to make sure there is central exposure.

Weaknesses

The methods section presented very few details, although some information could be extracted from the results and discussion section. There was no clear definition of "distress vocalization." Observations were not conducted blind to treatment level. The authors suggested that increased vocalization reflects increased distress. They cite Farabollini et al. (1999) as supporting evidence that BPA affects anxiety. However, they fail to indicate that Farabollini et al. (1999) concluded that BPA reduced anxiety in male rats, which would be contradictory to the findings of this paper.

Overall Assessment

Intracerebral injection into the brain resulted in very few behavioral and physiological effects. There were no effects on different measures of locomotion, brain, testis, liver, and kidney, or body weight. The only effect was an increase in the number of distress vocalizations over a 5-minute period. The route of exposure is not relevant to human exposures.

Sato, M., M. Shimada, and Y. Sato. 2001. The effects of prenatal exposure to ethinyl estradiol and bisphenol-A on the developing brain, reproductive organ and behaviour of mouse. Congenital Anomalies 41:187–193.

Strengths

The authors studied the effects of both BPA and ethinyl estradiol (EE) to enable a more direct test of the hypothesis that BPA adversely affects brain development and behavior as a result of its estrogenic effects. The effects of BPA and EE on brain development, diameter of the mammilothalamic tract, open-field behaviors, and diameter of seminiferous tubules were evaluated.

Weaknesses

It was unclear how animals were selected for different tests and whether there was an attempt to represent different litters in each group. The litter was not the statistical unit of comparison. No corrections were made for the multiple *t*-tests conducted, which included comparisons of BPA to control, EE to control, and BPA to EE. The rationale for evaluating the mammilothalamic tract and myelination was not discussed. The authors discussed statistically significant effects without examining the biological significance of the magnitude of the changes. For example, the only statistically significant effects between the BPA and control groups were grooming, defecation, and diameter of seminiferous tubules. However, the actual mean values were 0.33 incidents of grooming in the BPA group compared to 0.21 in the controls. Likewise for defecation, the difference is 1.34 fecal boli compared to 2.2 over a 3-minute period. In my opinion, these were not biologically meaningful effects. There were no clearly defined operational definitions for the neurobehavioral endpoints.

Overall Assessment

BPA decreased grooming and defecation during a 3-minute open-field examination of the mouse. These effects do not appear to be biologically significant adverse effects. BPA also increased the diameter of seminiferous tubules and decreased the number of cell layers in four to

six tubules. The effects of BPA on the seminiferous tubules should be evaluated relative to other reproduction studies to determine the biological relevance of this change.

Suzuki, T., K. Mizuo, H. Nakazawa, Y. Funae, S. Fushiki, S. Fukushima, T. Shirai, and M. Narita. 2003. Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D1 receptor-mediated action in mice: Enhancement of the methamphetamine-induced abuse state. Neuroscience 117(3):639–644.

Strengths

This study evaluated the effects of perinatal exposure to BPA in the diet on a) enhancement of methamphetamine-induced place preference, b) enhancement of methamphetamine-induced hyperlocomotion, c) increased sensitization of the effects of repeated doses of methamphetamine on hyperlocomotion, and d) increased dopamine D1 receptor production in the whole brain. The authors demonstrated that animals preferred the chamber that had been associated previously with methamphetamine (METH) doses, established a clear dose-response relationship, and reversed the effect by pre-treatment with a dopamine D1 receptor antagonist. The authors selected the dose of 0.5 METH, which did not increase preference for the drug-paired chamber and demonstrated that perinatal BPA exposure increased preference for the chamber that the animals associate with 0.5 METH. The authors also demonstrated that perinatal exposure to BPA enhanced the hyperlocomotion effects to single or repeated doses of METH. The authors evaluated the brains of the highest BPA dose group to understand the possible underlying neuropharmacologic mechanism for the behaviors measured.

Weaknesses

Authors did not estimate daily chemical consumption based on food consumption. The dietary levels were 0, 0.002, 0.5, and 2 mg BPA/g food. Pregnant dams were exposed to this diet from mating to weaning. Based on an assumption that mice eat 15 g food/100 g body weight/day (http://research.uiowa.edu/animal/?get=mouse), dose levels are approximately 0.03, 75, and 300 mg/kg-day body weight. The authors claimed that chronic exposure to BPA in females may predispose their children to a craving for psychostimulants. Much more evidence is required before such statements can be made responsibly.

Overall Assessment

This study provided preliminary evidence that perinatal exposures to BPA may have long-lasting effects on brain development at the receptor level that are unmasked by measuring behaviors following pharmacologic challenge. This study provides preliminary neurochemical evidence that perinatal exposure to BPA can potentiate the central dopamine D1 receptor-dependent neurotransmission, which is consistent with the behavioral effects. The human relevance of these findings regarding susceptibility to psychostimulant drug abuse cannot be determined based on the experimental evidence. The dose level at which effects were consistently measured was approximately 300 mg/kg-day. However, effects were also observed at 0.03 mg/kg-day. This study needs to be repeated by a different group of investigators.

UPDATED REVIEW OF THE DEVELOPMENTAL NEUROTOXICITY POTENTIAL OF BISPHENOL A (BPA)

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These 23 studies were selected based upon their recent publication or omission from previous reviews. They were evaluated and reviewed with respect to their scientific merit and potential relevance for regulatory decisions. Not all are directly related to the developmental neurotoxicity of Bisphenol A and two papers were reviews containing no original data.

Akingbemi et al. (2004)

This study evaluated the *in vivo* and *in vitro* effects of BPA. BPA gavage at 2.4 µg/kg/day from postnatal days 21-35 to Long-Evans rats decreased serum testosterone and luteinizing hormone levels when measured at PND 35; however, higher doses of BPA (10 µg/kg/day, 100 and 200 mg/kg/day) had no effects. Serum estradiol levels were decreased by BPA doses of 2.4 µg/kg/day to 100 mg/kg/day, but not 200 mg/kg/day. *Ex vivo* measures of Leydig cell production of testosterone indicated similar values in control and BPA preparations, but when these cells were incubated with luteinizing hormone, testosterone production was decreased in the 2.4 µg/kg/day group, but not higher dose groups. Further, this low dose (2.4 µg/kg/day) also down-regulated pituitary luteinizing hormone β expression, and upregulated estrogen receptor β expression. In male offspring of dams treated with 2.4 µg/kg/day from gestational day 12 through postnatal day 21, adult (postnatal day 90) body weight was increased while testis weight was decreased; however, serum luteinizing hormone and testosterone levels were unaffected. *Ex vivo* measures of Leydig cell production of testosterone indicated decreased values in the BPA group (2.4 µg/kg/day) as well as decreased levels when these cells were stimulated with luteinizing hormone. Similarly, *ex vivo* measures in rats treated on postnatal days 21-90 also indicated decreased testosterone production in the BPA group (2.4 µg/kg/day) as well as decreased levels when luteinizing hormone stimulated. However, body and testis weights of male rats treated on postnatal days 21-90 were unaffected as was serum testosterone level. Serum luteinizing hormone levels were elevated in these same rats (treated on postnatal days 21-90 with 2.4 µg/kg/day). *In vitro* studies of Leydig cell incubation with BPA, diethylstilbestrol (DES), or HPTE (a biologically active metabolite of methyoxychlor, an endocrine disrupter) indicated that only low concentrations of BPA (0.01 nM) reduced androgen biosynthesis; higher doses had no effect.

Scientific Merit:

Positive Features:

1. Rats were assigned to treatment groups by body weight randomization.
2. A wide range of BPA doses were used in the initial study (treatment from postnatal days 21-35).
3. Appropriate sample sizes were used (8-14 rats/endpoint studied).

Negative Features and Issues Impacting Interpretability:

1. A normal phytoestrogen-containing diet was used; however, the authors note that "all animals were exposed to the same levels of phytoestrogen because our feed intake was equivalent for control and BPA-treated rats".
2. Plastic cages and water bottles were used. The authors stated that "cages used in this study were washed, rinsed, and dried at least two times per week and were discarded once they began to get cloudy, and water bottles were cleaned daily". They further note that "repeated washing and rinsing are known to decrease the release of BPA from polycarbonate cages and water bottles" and cite Howdeshell et al. (2003) as evidence for this statement.
3. There is no indication that the study controlled for litter effects.

4. Subsequent to the initial study, only one dose of BPA was used (2.4 μg/kg/day).

Relevance to Humans:

Evaluation of endpoints that can be compared directly to humans (i.e., serum hormone levels) is particularly important.

Utility for Food Additive Regulatory Decisions:

If replicated, these effects on serum hormone levels and testosterone production by Leydig cells would be especially significant as a cause for concern.

Choi et al. (2007)

This study evaluated the effects of BPA on GABA-induced currents using patch clamp techniques. Hippocampal cells from the CA3 region were harvested from postnatal day 12-16 Wistar rats. The amplitude of BPA-induced currents increased with increasing concentrations of BPA and this was theorized to be mediated via $GABA_A$ receptor-Cl^- channel complexes.

Scientific Merit:

Positive Features:

1. Although patch clamp methodology is not a new technique, the study of BPA effects using this method is.

Negative Features and Issues Impacting Interpretability:

1. The authors state that it is "unlikely that BPA inhibits $GABA_A$ receptor-mediated Cl^- currents under physiological conditions" since the concentration required for this was very high.
2. There is no positive control such as DES or ethinyl estradiol.
3. There is no description of controlling for litter effects.
4. Sex of the rats from which the CA3 cells were harvested is not stated.
5. Type of caging, chow, water bottle are not stated.

Relevance to Humans:

As an *in vitro* study, it is difficult to make any direct extrapolation to humans. However, it might spur studies to examine further interactions of BPA with the GABAergic system.

Utility for Food Additive Regulatory Decisions:

The *in vitro* nature of this study makes it unlikely to be used directly for any regulatory decisions.

Facciolo et al. (2002)

This study evaluated the effects of BPA on somatostatin receptor (sst_2) binding in various brain regions. BPA (40 or 400 μg/kg/day) was administered orally to adult female Sprague-Dawley rats prior to mating and throughout gestation and lactation. Offspring were sacrificed on postnatal day 10 or 23 and binding affinities measured. Two types of binding affinities were identified (high and low) for this receptor in the presence of the high dose of BPA. These two binding affinities were differentially affected by the high dose of BPA and this varied across brain regions and with age at measurement (postnatal day 10 or 23). The low affinity state appeared to be more affected by BPA and in general, BPA induced diminished levels of the sst_2 receptor.

Scientific Merit:

Positive Features:

1. Investigation of BPA effects on a growth hormone is necessary given that many studies find BPA-induced body weight alterations.
2. The oral route of exposure mimics the human route.
3. Two doses of BPA (spanning an order of magnitude) were used.

Negative Features and Issues Impacting Interpretability:

1. Type of caging, chow and water bottles are not specified.
2. The sex of the rats in which binding affinities are measured is not stated.
3. It seems likely that the potential confound of litter was controlled, but this is not directly stated.
4. There is no positive control.
5. Even the low BPA dose is much higher than what is estimated for typical human exposure.

Relevance to Humans:

Presumably the somatostatin system is conserved in a similar manner in many mammalian species, including humans, and receptor type is common across many species. Use of developmental treatment only focuses on the major exposure period of concern in humans.

Utility for Food Additive Regulatory Decisions:

Until these results are replicated, it is not known how these data might be used for regulatory decisions. The low dose of BPA used here is much higher than that estimated for daily human consumption.

Fini et al. (2007)

This study utilizes a transgenic tadpole line to evaluate the effect of various compounds on thyroid function. This transgenic line contains an optimal thyroid hormone response gene. Tadpoles were exposed via aqueous incubation media. BPA had no effect by itself; however, when combined with T_3, BPA inhibited T_3 signaling.

Scientific Merit:

Positive Features:

1. Impaired thyroid function can interfere with brain development and cognitive function; thus, measurement of how BPA might influence thyroid function is a useful complement to other neurotoxicity measures.

2. Dose-dependent responses to T_3, TH agonists, antagonists, the classic thyroid disrupter methimazole, as well as BPA and tetrabromobisphenol A were measured yielding substantial information about the sensitivity of this testing method to detect compounds affecting thyroid function.

3. This testing paradigm (the transgenic tadpole line) yielded results in a shorter period of time than other similar paradigms and may be useful as a screening tool.

Negative Features and Issues Impacting Interpretability:

1. It is not clear how concentrations of BPA in the aqueous media relate to mammalian exposure.

Relevance to Humans:

The endpoints measured in this study involve elements common to all mammalian systems. However, until more is known about the effect of BPA on thyroid function *in vivo* in mammals, the data reported here cannot easily be interpreted.

Utility for Food Additive Regulatory Decisions:

Due to the use of a nonmammalian species, it is unlikely to be extremely helpful for regulatory decisions. However, the usefulness of this testing paradigm as a screening method for assessing the potential of endocrine disrupters to specifically affect thyroid function seems good.

Funabashi et al. (2001)

This study evaluated the effects of a single subcutaneous BPA injection (10 mg/rat) in adult ovariectomized female Wistar rats on mRNA levels of the progesterone receptor, preproenkephalin receptor and neurotensin receptor in the preoptic area, mediobasal hypothalamus, and anterior pituitary. BPA treatment significantly increased mRNA levels of the progesterone receptor in the medial preoptic area and the anterior pituitary. Both of these effects were similar to those of the positive control, 17-β-estradiol.

Scientific Merit:

Positive Features:

1. A positive control, 17-β-estradiol, was used to compare the effects of BPA.

Negative Features and Issues Impacting Interpretability:

1. Rats were subcutaneously injected with BPA which does not mimic the human oral route of exposure.

2. The BPA dose is 10 mg/rat. The body weight of the rats is not stated so as to allow an approximation of the dose on a mg/kg basis. Assuming an adult female Wistar rat weighs approximately 200 g, this translates into 40-50 mg/kg, a relatively high dose.

3. Type of caging, chow, and water bottle are not stated.
4. Only one dose of BPA was used.
5. There were no assessments of intact females, only ovariectomized.

Relevance to Humans:

Because of the difference in route of exposure, the high BPA dose used, no comparison to gonadally intact females, the relevance of these results is not easily discerned.

Utility for Food Additive Regulatory Decisions:

Due to the limitations stated above (high BPA dose, route of exposure, ovariectomized females), the results of this study are unlikely to be useful for regulatory decisions.

Funabashi et al. (2004)

This study is an extension of Funabashi et al. (2001). Here, the effects of a single subcutaneous BPA injection (10 mg/rat) in adult ovariectomized female Wistar rats on mRNA levels of the progesterone receptor in the frontal, parietal, temporal and occipital cortices were measured. BPA significantly increased levels in the frontal cortex and decreased levels in the temporal cortex, but had no effects on levels in the parietal cortex. The frontal cortical increase and temporal cortical decrease were apparent at 6, 12 and 24 hours post-injection.

Scientific Merit:

Positive Features:

1. As a follow-up to Funabashi et al. (2001), additional brain regions were measured here using the same BPA dose that affected mRNA levels in the preoptic area and anterior pituitary (they may even be the same animals).

Negative Features and Issues Impacting Interpretability:

1. Rats were subcutaneously injected with BPA which does not mimic the human oral route of exposure.
2. The BPA dose is 10 mg/rat. The body weight of the rats is not stated but the authors state this approximates 40 mg/kg. This is a very high dose.
3. Type of caging, chow, and water bottle are not stated.
4. Only one dose of BPA was used.
5. There were no assessments of intact females, only ovariectomized.

Relevance to Humans:

Because of the difference in route of exposure, the high BPA dose used, no comparison to gonadally intact females, these results are difficult to directly extrapolate to humans.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study, the results are unlikely to be useful for regulatory decisions.

Ghisari & Bonefeld-Jorgensen (2005)

This study evaluated the effects of BPA (as well as alkylphenols, pesticides, PCB metabolites and tetrabromobisphenol A) on the proliferation of GH3 cells, a rat pituitary tumor cell line used for studying effects on thyroid hormone actions. This cell line expresses intracellular thyroid hormone receptors and responds to thyroid hormone with proliferation. Although not as potent as T_3 at stimulating proliferation, BPA at various concentrations did increase proliferation; no concentration of BPA decreased proliferation. Similarly, in the presence of T_3, BPA increased proliferation.

Scientific Merit:

Positive Features:

1. A cytotoxicity assay was initially conducted using various concentrations of the tested chemicals and only those proliferation results that were obtained at concentrations that did not induce cytotoxicity were included in the final statistical analyses. Specifically, the concentrations used for statistical analyses were those that were not overtly toxic.

Negative Features and Issues Impacting Interpretability:

1. The *in vitro* nature of the study does not allow for easy extrapolation. It is not clear how the concentrations of BPA which altered thyroid function would relate to an oral dose.

Relevance to Humans:

As an *in vitro* study, it is difficult to make any direct extrapolation to humans. However, given the importance of thyroid hormones in brain development and cognitive function, these results demonstrate that a variety of endocrine disrupters, including BPA, may interact with thyroid system.

Utility for Food Additive Regulatory Decisions:

The *in vitro* nature of this study makes it unlikely to be used directly for any regulatory decisions. But the use of GH3 cells as a potential screening method for compounds which might alter thyroid hormone functioning could prove useful.

Jung et al. (2007)

This *in vitro* study describes the effect of BPA, TCDD, and Aroclor 1254 on thyroid function. The endpoints measured included antagonism of T_3 binding to the thyroid hormone receptor and T_3-mediated production of prolactin. BPA exhibited noncompetitive binding to thyroid hormone receptors and downregulated T_3-mediated prolactin production.

Scientific Merit:

Positive Features:

1. The authors note that TCDD, Aroclor 1254, and BPA have similar structures and that all are similar to T_3 and T_4, making a comparison of these compounds a good choice.
2. Impaired thyroid function can interfere with brain development and cognitive function; thus, measurement of how BPA might influence thyroid function is a useful complement to other measures of neurotoxicity.
3. Several concentrations of TCDD, Aroclor 1254 and BPA were used.

Negative Features and Issues Impacting Interpretability:

1. *In vitro* studies do not allow for easy extrapolation to animals. It is not clear how the concentrations of BPA which altered thyroid function would relate to an oral dose.

Relevance to Humans:

The endpoints measured in this study involve elements common to all mammalian systems. However, until more is known about the effect of BPA exposure on thyroid function *in vivo*, the data reported here cannot easily be interpreted.

Utility for Food Additive Regulatory Decisions:

Due to the *in vitro* nature of this study, it is unlikely to be helpful in this regard. However, its usefulness may be to spur additional studies *in vivo*.

Kiguchi et al. (2007)

Male Wistar rats were administered BPA (20 or 40 µg) intracisternally at postnatal day 5. At 8 weeks of age, baseline locomotor activity was measured and then again after injection with 1 or 5 mg/kg methylphenidate. At 11 weeks of age, jaw movements in response to injection with 1 mg/kg apomorphine were measured. BPA treatment had no effects on locomotor activity, either during the baseline period or in response to methylphenidate treatment. Nor did BPA treatment alter apomorphine-induced jaw movements differently than controls.

Scientific Merit:

Positive Features:

1. Observers scoring behaviors were blind to the treatment of the subjects.

Negative Features and Issues Impacting Interpretability:

1. Use of an intracisternal administration route is well outside the realm of comparability to human exposure.
2. Only males were examined.
3. Type of caging, chow, and water bottle are not specified.

4. Total number of litters is not stated, nor is it known if the litter was the unit of analysis.
5. No positive control was used.
6. Body weights are not reported.

Relevance to Humans:

The use of intracisternal administration of BPA makes it extremely difficult to determine how these doses might related to oral intake. Because no measured endpoint was affected, it is difficult to know if BPA had an effect at all.

Utility for Food Additive Regulatory Decisions:

The findings of this study using direct injections into the cerebrospinal fluid of neonatal rats are likely to have little direct safety assessment utility for supporting a regulatory decision on BPA.

Kim et al. (2007)

This study evaluated the effects of BPA exposure on immortalized neonatal mouse derived cerebellar cells (neural progenitor cells, NPCs). These cells only express the β form of the estrogen receptor. Only at relatively high concentrations (>100 μM) did BPA exposure decrease proliferation and only at even higher concentrations (>200 μM) did BPA exposure cause cytotoxicity. Newly generated cells (measured via BrdU) were decreased by exposure to 100 μM BPA; lower concentrations had no effect. The effects of BPA were similar to 17-β-estradiol in that concentrations of 100 μM or greater were necessary to decrease proliferation and cause cytotoxicity. Exposure to BPA at 400 μM decreased levels of phosphorylated extracellular signal-regulated protein kinases (ERK 1); however, BPA exposure elevated levels of phosphorylated c-Jun-N-Kinase (JNK) and did not alter levels of phosphorylated p38, another stress-related kinase.

Scientific Merit:

Positive Features:

1. BPA doses spanned more than one order of magnitude, allowing for a very thorough study of the dose-response.
2. Use of these NPCs which only express the β form of the estrogen receptor may help determine how BPA exerts its effects.
3. The authors note quite clearly that their results indicate BPA effects only occur at high concentrations.
4. A positive control (17-β-estradiol) was used.

Negative Features and Issues Impacting Interpretability:

1. The NPC cells were maintained in plastic culture flasks and these flasks could contain BPA.

Relevance to Humans:

Due to the *in vitro* nature of this study, it is difficult to know how the BPA concentrations might relate to human exposures.

Utility for Food Additive Regulatory Decisions:

Due to the *in vitro* nature of this study, it is unlikely to be helpful in this regard. However, its usefulness may be to spur additional studies further investigating the nature (e.g., estrogen receptor-mediated or via stress-related kinases) of the toxicity of BPA.

Kwon et al. (2000)

This study investigated the effects of three BPA doses administered via gavage between gestational day 11 and offspring postnatal day 20. Pregnant Sprague-Dawley rats were gavaged with vehicle, BPA or diethylstilbestrol (DES) and various endpoints measured during gestation. BPA at doses of 3.2, 32.0, or 320 mg/kg did not alter gestational or lactational weight, number of live pups, organ weights (liver, kidney, adrenal, ovary, uterus) of dams at sacrifice (at offspring weaning on postnatal day 21), or pup body weight at birth or postnatal day 7. Volume of the SDN-POA in postnatal day 10 female offspring was not altered by 320 mg/kg BPA but was increased in females of dams treated with 15 µg/kg DES. Age at vaginal opening, first ovulation, and estrous cyclicity were not affected by any dose of BPA nor was lordosis response altered. Organ weights (testes, epididymis, seminal vesicle, prostate lobes) of male offspring when sacrificed at 6 months of age were not altered by any dose of BPA.

Scientific Merit:

Positive Features:

1. The oral route of BPA exposure best mimics human exposure.
2. Three doses of BPA were used, allowing for dose-response relationships to be examined.
3. Use of DES as a positive control in endocrine disrupter studies is quite common and allows assessment of the sensitivity of the endpoints to a classic estrogen disrupter.
4. The litter was used as the unit of analysis.
5. Glass water bottles were used.
6. Each treatment group contained a sufficient n (8 dams/group).
7. A phytoestrogen-free diet (NIH-07) was used.
8. Pregnant dams were assigned to treatment groups via randomization of body weights.

Negative Features and Issues Impacting Interpretability:

1. Animals were housed in polycarbonate cages.
2. Different endpoints were measured in male and female offspring (e.g., SDN-POA volume measured only in females).
3. The dose of DES, although affecting SDN-POA volume in females, had very few effects.

4. SDN-POA volume was measured only in females and then only on postnatal day 10, although treatment continued for another 10 days after this.

Relevance to Humans:

This study used the most common laboratory rat strain (Sprague-Dawley) allowing vast comparisons with the literature. The route of exposure via gavage approximates the human scenario. The endpoints investigated are common to many mammalian species.

Utility for Food Additive Regulatory Decisions:

The lowest dose of BPA used (3.2 mg/kg/day) is much higher than that estimated for humans. Even the highest dose used (320 mg/kg) had few effects. Thus, the sensitivity of these endpoints in this particular lab is questionable. As such, the data are unlikely to be useful for regulatory decisions.

Le et al. (2008)

This study evaluated the factors affecting BPA release from new and used polycarbonate or high-density polyethylene water bottles. The release of BPA into water stored in polycarbonate bottles was measurable (ranging 0.25-0.79 ng/ml in concentration) and similar in both new and used bottles after 3 days of water storage at room temperature and this increased with additional days of storage. On the other hand, BPA was almost undetectable in water stored for a similar amount of time in high-density polyethylene bottles. Heating water before placing into polycarbonate caused increased BPA release. When water containing BPA (from polycarbonate bottles) or 17-β-estradiol was added to an *in vitro* assay containing cerebellar granule cells from neonatal Sprague-Dawley rats, the effects on lactate dehydrogenase release were nearly identical at the same concentrations of BPA and 17-β-estradiol. Both BPA and 17-β-estradiol at concentrations of 0.01-1.0 nM increased lactate dehydrogenase release.

Scientific Merit:

Positive Features:

1. The "used" polycarbonate water bottles in this study were obtained from consumers that had been using the bottles for several years. It is difficult to imagine a more directly relevant exposure source for humans. New polycarbonate and high-density polyethylene bottles were purchased from an outdoor equipment store.
2. The methods for water storage over extended time (up to 7 days) involved rotating the bottles to mimic the water motion during typical consumer usage.
3. The effects of heated water on BPA release were studied as this is likely to be the method consumers used to "wash" their bottles.
4. The water that had been stored in the polycarbonate bottles, which now contained BPA, was the compound added to cultured cells *in vitro*. As a control, BPA (from Sigma-Aldrich) and 17-β-estradiol were added to other samples. Thus, comparisons of BPA obtained in a manner directly related to consumer use, commercial

grade BPA and 17-β-estradiol could be made. This allowed an estimation of the "bioactivity" of the BPA in the water bottles using an *in vitro* system.

Negative Features and Issues Impacting Interpretability:

1. This is an *in vitro* study and, therefore, provides little opportunity for extrapolation of findings to human exposure.

Relevance to Humans:

For purposes of estimating likely human intake from the use of polycarbonate bottles, this study has high relevance. It would be easy to estimate the amount of BPA consumed by a human with a new or used polycarbonate water bottle given amount of water consumed and time that the water remains in the bottle. Because the authors purchased water bottles directly from a store which routinely (likely, daily) sells such products to the consumer, the relevance cannot be overstated. However, the biological relevance of the effects of the BPA on neonatal rat cerebellar neurons *in vitro* is not known and the concentrations of BPA utilized are difficult to relate to human exposures since concentrations reaching brain tissues in humans after oral exposures are not likely known.

Utility for Food Additive Regulatory Decisions:

While it has been known that BPA can migrate from polycarbonate water bottles, this migration has been described to occur in used or scratched bottles. This study provides evidence that such migration can occur in new bottles at a very similar rate as in used bottles. Further, placing heated water into these bottles (as might occur when baby bottles are sterilized or when a hot beverage is stored) increases the migration rate.

Lee et al. (2007)

This study evaluated the *in vitro* responses of PC12 or cortical cells derived from brains of day 18 rat embryos to BPA. BPA dose-dependently inhibited the viability of PC12 cells. Cortical neuronal cell viability was slightly increased after incubation with the lowest concentration of BPA (10 μM), but significantly declined at higher concentrations. However, these effects on PC12 and neuronal cell cultures did not appear related to the estrogen receptor since pretreatment of the cultures with estrogen receptor antagonists did not abolish the BPA-induced decrease in viability. Further, viability was not significantly different in PC12 cells over-expressing either estrogen receptor α or β, indicating that BPA toxicity is not selective for either type of receptor or its toxicity in this model is unrelated to its interaction with estrogen receptors. BPA exposure caused PC12 cells to activate the mitogen activation protein (MAP) kinase in a dose-dependent manner.

Scientific Merit:

Positive Features:

1. Up to eight concentrations of BPA were used to thoroughly investigate dose-response effects.

2. The critical involvement of the estrogen receptor for the neurotoxic effects was studied in a well-thought out manner.

Negative Features and Issues Impacting Interpretability:

1. No positive control was used, except in the portion comparing BPA effects to those of 17-β-estradiol.

Relevance to Humans:

Due to the *in vitro* nature of this study, it is difficult to know how the BPA concentrations used here might relate to human exposure. The authors cite a study in which the concentration of BPA in amniotic fluid is approximately 40 nM and note that the BPA concentrations used in their study were 100-2000 times higher. They note that BPA concentrations in human blood could reach the μM range "in the case of high-dose oral supplementation or intravenous therapy". It is not clear what type of oral supplementation or iv therapy would provide these high doses of BPA.

Utility for Food Additive Regulatory Decisions:

Due to the *in vitro* nature of this study, it is unlikely to be helpful in this regard. However, its usefulness may be to spur additional studies further investigating the nature (e.g., estrogen receptor-mediated or otherwise) of the toxicity of BPA.

Leranth et al. (2008)

This study evaluated the ability of the medial prefrontal cortex and hippocampus of intact and castrated male rats treated with BPA to respond to testosterone. Adult male Sprague-Dawley rats were sham-operated or castrated and subcutaneously injected with 300 μg/kg BPA for 4 consecutive days. Brains were removed 30 minutes after the last BPA injection and number of asymmetric spine synapses in the medial prefrontal cortex and hippocampus were measured. In gonadally intact rats, BPA treatment significantly reduced the number of spine synapses in the cortical area as well as the CA1 area of the hippocampus. BPA treatment also increased the density of astroglia processes in both areas. In castrated rats, BPA treatment completely eliminated the synaptogenic response to testosterone. Specifically, in castrated rats, testosterone treatment increased the number of spine synapses in the medical prefrontal cortex and CA1 area of the hippocampus; however, castrated rats treated with BPA do not show this increase in either brain region.

Scientific Merit:

Positive Features:

1. Observers were blind to the treatment conditions of the animals.
2. The authors are familiar with these types of measurements and were able to use previous data from their lab to conduct a power analysis determining the appropriate n required.

Negative Features and Issues Impacting Interpretability:

1. Rats were fed "regular rat chow", not a phytoestrogen-free diet.
2. Water bottle and caging type are not specified.
3. No positive control was used.
4. Rats were obtained from the supplier as adults so it is unclear if any may have been littermates.
5. BPA treatment was via subcutaneous injections, rather than the oral route.

Relevance to Humans:

Use of a mammalian species is useful for extrapolation. The endpoints measured here are similar, if not identical, in humans. However, the dose is much higher than that estimated for daily oral exposure in humans. More information regarding dose-response effects on these endpoints is needed.

Utility for Food Additive Regulatory Decisions:

With additional dose-response information, the results of this study could be useful. Still, the BPA exposure here occurred during adulthood, not developmentally, and it is developmental exposure that appears to be of concern.

Miyagawa et al. (2007)

This study evaluated the effects of dietary exposure to BPA on several different behaviors in mice. Female mice consumed a control diet or one of two dietary concentrations of BPA (30 ng/g or 2 mg/g) from mating until offspring weaning. Male offspring were assessed for anxiety (light-dark paradigm, elevated plus maze), motor coordination (Rotarod test), and passive avoidance. Their brains were assessed for density of hippocampal ChAT labeling. Dietary exposure to BPA had no effects on anxiety behavior or motor coordination. Passive avoidance retention was impaired by exposure to both concentrations of BPA in the diet, implying a BPA-induced effect on learning/memory. ChAT immunoreactivity was significantly reduced in the hippocampus by both concentrations of BPA diets.

Scientific Merit:

Positive Features:

1. BPA exposure was oral, mimicking the human route of exposure.
2. The behaviors assessed and the methodology used are typical of those reported in the literature, allowing cross-study comparisons.

Negative Features and Issues Impacting Interpretability:

1. No positive control was included.
2. Only male mice were studied.
3. It is not clear that the litter effect was controlled. The authors state that they "randomly selected a few pups per litter and housed to undergo the behavioral tests. To obtain unbiased results, we appropriately distributed mice for each behavioral study". While this seems confusing given the wording, it does not seem to take account of litter effects.

4. Type of caging, chow, and water bottle are not specified.
5. It is not stated that the observers of the behaviors were blind to treatment conditions.
6. There is no daily food intake data reported so it is not possible to estimate the daily BPA intake.

Relevance to Humans:

The authors do not provide food intake data so it is difficult to determine the daily intake of BPA. These data are essential in order to evaluate the relevance of these dietary concentrations to human intake.

Utility for Food Additive Regulatory Decisions:

This study provides evidence that BPA does not globally alter behavior after developmental exposure; that is, its effects appear to be functionally specific. However, the negative features of this study (listed above) should be considered in the overall impact of these results.

Monje et al. (2007)

This study examined female Wistar rats treated on postnatal days 1-7 with different doses of BPA or a positive control (diethylstilbestrol, DES). Female pups treated with 0.5 mg/kg BPA via subcutaneous injections and sacrificed on postnatal day 8 or 21 had increased estrogen receptor α mRNA levels as well as increased estrogen receptor α protein expression in the preoptic area. However, mRNA levels in female pups treated with 20 mg/kg BPA depended on age at measurement: on postnatal day 8, levels were significantly lower than control while on postnatal day 21, levels were significantly higher. Postnatal day 21 serum levels of estradiol did not differ between experimental groups.

Scientific Merit:

Positive Features:

1. Use of DES as a positive control is an advantage. DES treatment (0.02 mg/kg) caused significant effects in mRNA levels, but not on anogenital distance or serum estradiol levels.
2. Water was provided in glass bottles.
3. Two doses of BPA were used (0.05 and 20.0 mg/kg).

Negative Features and Issues Impacting Interpretability:

1. Use of a subcutaneous route of exposure does not mimic human exposure.
2. The authors state that on the day of birth (prior to treatment), pups were cross-fostered and that this "allowed us to minimize the use of siblings to avoid potential litter effects". If there were only 8-10 total litters and each litter was assigned to one of the four treatment groups (control, low dose BPA, high dose BPA, DES), then it is unclear how the authors state that "the number of animals per group at each time point evaluated was 12-14" without the use of same-treatment siblings.

3. There are four treatment groups in this study which would suggest that a one-way ANOVA is the best statistical analysis. Instead, this study conducted multiple t-tests for each endpoint. For example, for mRNA levels at postnatal day 8, four different t-tests were conducted: low dose BPA compared to control; high dose BPA compared to control; DES was compared to control; and finally, control males compared to control females. This type of comparison easily inflates the chances of finding a significant effect.

4. The authors measured the identical endpoints in all control and treated females and in control male pups as well, but there were no treated male groups. Since there were significant sex effects in mRNA levels between control males and control females, it is not clear why the study did not treat males as well.

5. The "pellet laboratory chow" is from Cooperacion, an Argentinian company and is not stated to be phytoestrogen-free.

6. Caging type is not specified.

Relevance to Humans:

Similar sexually dimorphic hypothalamic brain regions have been described in humans; however, use of a non-oral route of exposure lessens the degree of extrapolation to humans. The restricted period of treatment (postnatal days 1-7 only) would approximate the last trimester of human pregnancy and this is not be particularly applicable to human exposure paradigms. It will be important to know if males are similarly affected and if the effects persist into adulthood.

Utility for Food Additive Regulatory Decisions:

Due to the limitations of this study and the interpretation uncertainties, this study is unlikely to be useful for supporting a food additive regulatory decision on BPA.

Nagao et al. (1999)

This study examined the effect of BPA or estradiol benzoate injected subcutaneously from postnatal day 1-5 in male and female Sprague-Dawley rats. BPA (at the only dose examined of 300 μg/kg/day) had few effects on body weight, developmental endpoints (preputial separation, testicular descent, vaginal opening), reproductive ability, male sexual behavior, or adult volume of the SDN-POA. Estradiol benzoate treatment altered several endpoints.

Scientific Merit:

Positive Features:

1. Metal caging was used, rather than polycarbonate.

2. The use of estradiol benzoate as a positive control allows for evaluation of the sensitivity of the endpoints.

3. Both sexes were assessed.

Negative Features and Issues Impacting Interpretability:

1. Only one dose of BPA was used, limiting any interpretation of dose-response relationships.

2. A subcutaneous route of exposure makes difficult any comparisons with the oral route of humans.

3. The rat chow is described as CE-2 from Clea Japan. It is not stated if this is a phytoestrogen-free chow.

4. It is not at all clear how many litters were used, whether all pups in a litter were treated similarly (all BPA or mixed treatments), or whether the litter was used as the unit of analysis.

5. There is no statement that observers of the behaviors (i.e., male sexual behavior) were blind to the treatment conditions of the animals.

Relevance to Humans:

Use of a mammalian species is useful for extrapolation. However, the dose (300 µg/kg/day via subcutaneous injections) is much higher than that estimated for daily oral exposure in humans. More information regarding dose-response effects on these endpoints is needed. This study has little, if any, direct safety assessment utility for supporting a food additive regulatory decision on BPA.

Utility for Food Additive Regulatory Decisions:

It is difficult to compare the dose used in a subcutaneous route of exposure with the oral exposure route in humans. If they are at all comparable, the single BPA dose used here is much higher than estimated for humans.

Nishizawa et al. (2003)

This study evaluated the effects of BPA treatment on the expression of retinoid receptors (retinoic acid receptor α (RARα) and retinoid X receptor α (RXRα)) in the cerebrum, cerebella, and gonads of mouse embryos. Pregnant mice were orally treated with BPA (2 µg/kg/day) beginning on post-conception day 6.5 and embryos were harvested at various gestational ages. Effects of BPA seemed inconsistent.

Scientific Merit:

Positive Features:

1. Both male and female embryos were evaluated.

Negative Features and Issues Impacting Interpretability:

1. Only one dose of BPA was used (2 µg/kg/day).
2. No positive control was used.
3. The effects of BPA seem sporadic. For example, BPA decreased expression of RARα in the cerebrum at 14.5 days post-conception, but not 12.5, 16.5, or 18.5 days post-conception.
4. A standard mouse diet was used, presumably phytoestrogen rich.
5. Caging and water bottle type is not specified.
6. It is not specified that the litter was used as the unit of analysis.

Relevance to Humans:

Because the changes in expression levels of RARα and RXRα are likely to be different between human and murine development, it is unknown how BPA exposure during post-conception days 6.5-14.5 which altered cerebral levels of expression of RARα would translate to humans. There is no justification for assessing retinoic acid receptors in BPA-treated mice.

Utility for Food Additive Regulatory Decisions:

Because of the limitations of this study, it is likely to have little relevance for supporting a food additive regulatory decision on BPA.

Ogiue-Ikeda et al. (2008)

This is a review of the rapid modulation of hippocampal synaptic plasticity by endogenous estrogens with a very brief review of similar endpoints using endocrine disrupters (i.e., BPA, diethylstilbestrol, nonylphenol, octylphenol). Several articles are cited in which perfusion with BPA enhanced long-term depression in the CA1 and CA3 areas of the hippocampus as well as increased spine density which the authors state are similar to the effects of estradiol. Such results seem to conflict with those of Leranth et al. (2008) (also reviewed here).

Scientific Merit:

Positive Features:

1. The review of the effects of estradiol on long-term depression and long-term potentiation as well as spine density is very well-written.

Negative Features and Issues Impacting Interpretability:

None.

Relevance to Humans:

Long-term depression and potentiation are common to most mammalian species. Still, the concentrations of BPA used *in vitro* cannot easily be compared to human estimated intake.

Utility for Food Additive Regulatory Decisions:

As a review, there are no original data presented here.

Panzica et al. (2007)

This is a review of the effects of endocrine disrupters (BPA, methoxychlor, diethylstilbestrol, and genistein) in several species (rat, mouse, and Japanese quail) with an emphasis on sexually dimorphic behaviors (in rodents, exploration and anxiety behavior).

Scientific Merit:

Positive Features:

1. The authors present brief overview of the effects of endogenous hormones on brain development.
2. The authors describe the method used in their lab for bolus oral dosing without the use of gavage: mice are trained to spontaneously consume a small volume of corn oil (with or without the test compound) from a modified syringe.

Negative Features and Issues Impacting Interpretability:

None.

Relevance to Humans:

It is not clear how data derived from birds (i.e., the Japanese quail) might relate to human effects.

Utility for Food Additive Regulatory Decisions:

As a review, there are no original data presented here.

Patisaul & Bateman (2008)

Male Long-Evans rats were injected with 50 μg/kg BPA, 50 μg estradiol benzoate, or an estrogen receptor α agonist, an estrogen receptor β agonist, or equol (an endocrine disrupter) for four consecutive days beginning on the day of birth. At adulthood, anxiety and aggressive behavior were measured. BPA increased adult body weight and increased anxiety-like behavior.

Scientific Merit:

Positive Features:

1. A phytoestrogen-free diet was used.
2. An attempt was made to determine if the effects of BPA were mediated via the ER α or β by using an agonist specific to each.
3. Estradiol benzoate served as a positive control.
4. Observers scoring the behavioral tests were blind to the treatment conditions of the animals.

Negative Features and Issues Impacting Interpretability:

1. Exposure was subcutaneous injection and it is not known how this compares with the oral exposure of humans, although the authors state that the BPA injection dose of 50 μg/kg is equivalent to the LOAEL established by the FDA.
2. Type of caging and water bottle are not stated.
3. As the "intruder" in the resident-intruder test, a different strain of rat was used (Wistar).
4. The total number of litters at birth was 8 and the males in these litters were cross-fostered such that only 2 males/litter remained with their biological dam. All offspring within the litter were similarly treated and this resulted in 2 control litters and 2 BPA litters. It is not clear if litter was the experimental unit of analysis or if the authors

believe this cross-fostering took care of the litter effect (it would not). It is likely that litter is a potential confound since if not, there would only be 2 BPA subjects, 2 control subjects, etc. and this is probably not the case.

5. Only males were examined.

Relevance to Humans:

A mammalian species was used and the behaviors assessed are very common in the literature. Still the lack of an oral route of dosing complicates the interpretation.

Utility for Food Additive Regulatory Decisions:

Until more is known regarding the bioavailability of subcutaneous injections compared to oral doses, it is not clear how the BPA dose used here related to human exposure.

Seiwa et al. (2004)

This study examined the effects of BPA on precursor cells of oligodendrocytes, the glial cell responsible for myelin formation in the central nervous system. Precursor cells were obtained from embryonic day 17 mice and treated with BPA. When high concentrations of BPA were added to the cell culture medium, the cells failed to differentiate into mature oligodendrocytes. However, over 95% of the cells survived at concentration of BPA up to 10^{-5}M. Further, this effect appeared to be mediated by the β1 version of the thyroid hormone receptor and not the α version.

Scientific Merit:

Positive Features:

1. Investigation of the interactions of thyroid hormones and BPA provides additional evidence of the mechanisms by which BPA might cause developmental neurotoxicity.

Negative Features and Issues Impacting Interpretability:

1. There is no description of the type of chow, caging, or water bottle used for the pregnant mice.
2. The sex of the embryos from which the precursor cells were obtained is not specified.
3. There is no description of control for litter effects.

Relevance to Humans:

As an *in vitro* study, it is difficult to make any direct extrapolation to humans. However, given the importance of thyroid hormones in brain development and cognitive function, these results demonstrate that a variety of endocrine disrupters can interfere with thyroid function.

Utility for Food Additive Regulatory Decisions:

The *in vitro* nature of this study makes it unlikely to be used directly for any regulatory decisions. However, the results demonstrate that cells other than neurons are likely to be affected by BPA treatment and this will likely spur research into the effects of BPA on glial cell development/survival.

Takagi et al. (2004)

This study examined several dietary doses of BPA or nonylphenol (another endocrine disrupter) during gestation and lactation in Sprague-Dawley rats. Anogenital distance, measured on PND 2, was not affected by any BPA dose even though the highest dose of 3000 ppm decreased PND 2 body weight. Body weight after PND 2 was only affected by the highest BPA dose (3000 ppm, estimated at 230-385 mg/kg/day). The positive control (ethinyl estradiol) affected gestational weight gain, maternal food intake, and offspring body weight as well as facilitated vaginal opening in female offspring and delayed preputial separation in male offspring. Vaginal opening, preputial separation and estrous cyclicity were not affected by BPA exposure at any dose. Adult organ weights were not affected by BPA exposure at any dose, although body weight of adult male offspring was less than controls (there were no body weight differences between adult female offspring groups). BPA exposure at any dose did not affect volume of the SDN-POA.

Scientific Merit:

Positive Features:

1. Dietary route of exposure is most similar to human exposure.
2. Use of a soy-free diet (stated to be similar to NIH-07 diet) restricts the effects to the compounds studied.
3. Three different dietary doses of BPA, three different dietary doses of nonylphenol, and a single dose of ethinyl estradiol (as a positive control) were used. The highest BPA dose decreased gestational weight gain and affected body weight of the offspring, indicating that such measures could be altered by BPA.
4. Dietary exposure began on gestational day 15 and continued until offspring postnatal day 10, a time encompassing fetal and early postnatal brain development.
5. Endpoints included those typical and necessary in a study of this sort: anogenital distance, age at vaginal opening or preputial separation, estrous cyclicity, and volumetric measurement of the SDN-POA.
6. Litter was used as the unit of statistical analysis.
7. The expected sexual dimorphism in volume of the SDN-POA was apparent in the control groups.

Negative Features and Issues Impacting Interpretability:

1. Rats were housed in polycarbonate cages.
2. Only 5-6 dams/treatment group were used.
3. Anogenital distance and SDN-POA volume were not measured in the positive control (ethinyl estradiol) group.

Relevance to Humans:

This study used the most common laboratory rat strain (Sprague-Dawley) allowing vast comparisons with the literature. The route of exposure via diet approximates the human scenario. The endpoints investigated are common to many mammalian species.

Utility for Food Additive Regulatory Decisions:

The lowest concentration of BPA used (60 ppm) was calculated to provide 5100-8500 μg/kg/day which is much higher than that estimated for humans.