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Developmental Neurotoxicity Study of Dietary Bisphenol A in Sprague-Dawley Rats

Donald G. Stump,*[,1] Melissa J. Beck,* Ann Radovsky,* Robert H. Garman,† Lester L. Freshwater,‡ Larry P. Sheets,§ M. Sue Marty,¶ John M. Waechter, Jr,¶ Stephen S. Dimond,|| John P. Van Miller,||| Ronald N. Shiotsuka,|||| Dieter Beyer,# Anne H. Chappelle,** and Steven G. Hentges††

**Developmental and Reproductive Toxicology, WIL Research Laboratories, LLC, Ashland, Ohio 44805-8946; †Consultants in Veterinary Pathology, Inc., Murrysville, Pennsylvania 15668; ‡BioSTAT Consultants, Inc., Portage, Michigan 49024; §Regulatory Toxicology, Bayer CropScience LP, Research Triangle Park, North Carolina 27709; ¶Toxicology and Environmental Research and Consulting, The Dow Chemical Co., Midland, Michigan 48674; ||Environmental Health and Safety, SABIC Innovative Plastics, Pittsfield, Massachusetts 01201; |||Toxicology Regulatory Services, Inc., Charlottesville, Virginia 22911; ||||Bayer MaterialScience, Pittsburgh, Pennsylvania 15205; #Bayer Schering Pharma AG, Wuppertal, Germany D 42096; **Sunoco, Inc., Philadelphia, Pennsylvania 19103; and ††Polycarbonate Business Unit, American Chemistry Council, Arlington, Virginia 22209*

[1] To whom correspondence should be addressed at Developmental and Reproductive Toxicology, WIL Research Laboratories, LLC, 1407 Ashland, Ohio 44805-8946. Fax: (419) 289-3650. E-mail: dstump@wilresearch.com.

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This study was conducted to determine the potential of bisphenol A (BPA) to induce functional and/or morphological effects to the nervous system of F_1 offspring from dietary exposure during gestation and lactation according to the Organization for Economic Cooperation and Development and U.S. Environmental Protection Agency guidelines for the study of developmental neurotoxicity. BPA was offered to female Sprague-Dawley Crl:CD (SD) rats (24 per dose group) and their litters at dietary concentrations of 0 (control), 0.15, 1.5, 75, 750, and 2250 ppm daily from gestation day 0 through lactation day 21. F_1 offspring were evaluated using the following tests: detailed clinical observations (postnatal days [PNDs] 4, 11, 21, 35, 45, and 60), auditory startle (PNDs 20 and 60), motor activity (PNDs 13, 17, 21, and 61), learning and memory using the Biel water maze (PNDs 22 and 62), and brain and nervous system neuropathology and brain morphometry (PNDs 21 and 72). For F_1 offspring, there were no treatment-related neurobehavioral effects, nor was there evidence of neuropathology or effects on brain morphometry. Based on maternal and offspring body weight reductions, the no-observed-adverse-effect level (NOAEL) for systemic toxicity was 75 ppm (5.85 and 13.1 mg/kg/day during gestation and lactation, respectively), with no treatment-related effects at lower doses or nonmonotonic dose responses observed for any parameter. There was no evidence that BPA is a developmental neurotoxicant in rats, and the NOAEL for developmental neurotoxicity was 2250 ppm, the highest dose tested (164 and 410 mg/kg/day during gestation and lactation, respectively).

Key Words: **bisphenol A; CAS No. 80-05-7; developmental neurotoxicity; OECD 426; neurobehavior; learning and memory; neuropathology; morphometry.**

Bisphenol A (BPA) is a high–production volume chemical used primarily to manufacture polycarbonate plastics and epoxy resins, and is considered to have weak estrogen-like properties. Human exposure to low doses (typically <0.1 μg/kg body weight/day; Dekant and Völkel, 2008) of BPA occurs principally through food contact use of these materials (e.g., polycarbonate plastic is used in plastic bottles and epoxy resins are used to coat food and beverage containers; EFSA, 2006; FDA, 2008). Some expert panel and government reviews (Chapin *et al.*, 2008; FDA, 2008; Health Canada, 2008) of the extensive BPA literature indicated the need for the development of additional data to examine the potential of dietary BPA to produce functional or morphological effects on the developing nervous system. In a previous multigeneration reproduction study that incorporated behavioral measures following low-dose exposure, BPA had no effects on F_1 or F_2 developmental landmarks, open-field behavior, cognitive tasks (i.e., T-maze and Biel maze), or brain weights at doses ranging from 0.2 to 200 μg/kg/day (Ema *et al.*, 2001). However, a developmental neurotoxicity study (DNT) conducted according to regulatory guidelines has not been published.

Therefore, the present study was conducted with BPA oral exposure in rats in compliance with the most current developmental neurotoxicity guidelines, Organization for Economic Cooperation and Development (OECD) Test Guideline (TG) 426 (OECD, 2007a) and U.S. Environmental Protection Agency (EPA) OPPTS Guideline 870.6300 (U.S. EPA, 1998), and in compliance with Good Laboratory Practice (GLP) principles (OECD, 1998, 2002; U.S. EPA, 1989) to examine widely accepted and validated neurobehavioral and neuropathological end points. The study design exceeded the EPA and OECD guidelines by evaluation, and external peer review, of neuropathology and brain morphometry for all

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groups rather than the high-dose and control groups only, and in the use of five (vs. three) dietary levels of BPA, ranging from very low concentrations to concentrations sufficient to achieve maternal toxicity. As specified in the cited guidelines, positive control data, using reference chemicals in Sprague-Dawley rats, established the reliability and sensitivity of the neurotoxicology test methods under the conditions used for the study. The study used the rat, the preferred test species for DNT studies according to the guidelines; the Sprague-Dawley strain was used as there was a large database with historical control (untreated) and positive control data for DNT end points.

Dietary concentrations of BPA in this study (0, 0.15, 1.5, 75, 750, and 2250 ppm; mean target doses ~0, 0.01, 0.1, 5, 50, and 150 mg/kg/day) were selected to provide doses that spanned the range from low doses used in some published studies reporting developmental neurotoxicity (Chapin *et al.*, 2008) to a high dose that was anticipated to result in systemic toxicity in the pregnant rat. This range provided for evaluation of potential low-dose effects as well as potential nonmonotonic dose-response relationships. The high dose was selected to produce maternal and neonatal body weight effects without resulting in developmental delays that would significantly impact the neurobehavioral measurements (specified by the guideline). The F_1 animals in the present study were exposed to the test substance *in utero* (Domoradzki *et al.*, 2003), as well as via the milk while nursing (Snyder *et al.*, 2000) and via direct consumption of the diet during the third week of the lactation period. To maximize exposure, the diets were maintained at a constant concentration (i.e., diet was not adjusted to provide a consistent milligram per kilogram per day dose of BPA) during lactation, although increased feed consumption by the dam (to support milk production) results in a higher dose during this period.

The objectives of this study were to determine the potential of BPA, administered in the feed to Sprague-Dawley rats, to induce functional and/or morphological effects in the nervous system that may arise in the offspring from exposure of the mother during pregnancy and lactation. The results of this guideline-based study are expected to be useful for human health risk assessment as these end points are generally recognized as relevant to neurodevelopment in humans.

MATERIALS AND METHODS

The present study was conducted in compliance with OECD (TG 426) and U.S. EPA OPPTS (870.6300) guidelines and followed U.S. EPA and OECD principles of GLPs. The study design exceeded the guideline requirements as there were more dose groups, to accommodate the typical dose range (i.e., to induce some maternal toxicity and establish a no-observed-adverse-effect level [NOAEL]), as well as use of a low-dose range. In addition, neuropathology and morphometry were conducted for animals in all groups to ensure the evaluation of potential low-dose effects; the guidelines specify examination of only control and high-dose tissues unless there is evidence of a treatment-related lesion in high-dose animals.

Test Substance and Dose Formulations

BPA (4,4′-isopropylidene-2-diphenol; CAS No. 80-05-7) was received as a white granular solid (purity >99.5%) from Acros Organics NV (Fairlawn, NJ). The feed used for preparation of the test diets was Certified Ground Rodent LabDiet 5002 (PMI Nutrition International, LLC, St Louis, MO). Diets (0, 0.15, 1.5, 75, 750, and 2250 ppm BPA in feed) were formulated using stock solutions of BPA dissolved in acetone. For the 0.15- and 1.5-ppm dose groups, the BPA solution was added to the feed and blended. For the 75-, 750-, and 2250-ppm dose groups, a concentrated premix was prepared using the BPA acetone stock solutions, and the test diets were prepared by dilution of this premix. For the control diet, a volume of acetone equal to the largest amount added for the test diets was added. The homogeneity and stability of BPA in the diets at concentrations of 0.010 and 7500 ppm were established. The test diets were prepared at least biweekly and stored at room temperature or in a freezer in accordance with stability data.

BPA diet concentrations were confirmed by analysis of all batches of feed that were presented to the animals with a limit of detection and a limit of quantitation (LOQ) of 0.0032 and 0.010 ppm, respectively. Only formulations that satisfied the acceptability standard (mean concentration within ±15% of the target concentration) were considered acceptable for use. The analytical range for all diets used was 86.6–113% of the target concentration for BPA. All analyses of control diet used in the study were below the LOQ.

Animals and Husbandry

One hundred eighty (180) virgin female Sprague-Dawley Crl:CD (SD) rats (Charles River Laboratories, Inc., Raleigh, NC) were received in two shipments, 90 per shipment, 4 weeks apart. The animals were approximately 70 days old upon receipt. Animals from the first and second shipments were designated as cohorts 1 and 2, respectively. Use of cohorts was necessary to accommodate the logistics of data collection due to the number and size of the dose groups. Each cohort consisted of 12 mated females per dose group. Sexually mature resident males of the same strain and source, maintained exclusively for mating, were used to induce pregnancy. Each male was used to sire only one litter. Each female judged to be in good health and meeting the acceptable body weight requirement (minimum of 220 g) was paired with a male for mating at approximately 12 weeks of age.

Each F_0 female was uniquely identified by a Monel (National Band and Tag Co., Newport, KY) metal ear tag. The F_1 offspring selected to continue on study were identified by foot markings at the end of parturition and uniquely identified by a Monel metal ear tag following weaning.

All F_0 females and their offspring were housed in the same room from arrival to termination; the animals from the two cohorts were housed in separate similar environmentally controlled rooms. The F_0 females were housed individually in clean suspended wire mesh stainless steel cages from arrival until gestation day (GD) 18. On GD 18, females were transferred to polyphenylsulfone RaTEMP thermoplastic rat cages (Allentown, Inc., Allentown, NJ) with ground corncob nesting material (Bed O'Cobs; The Andersons, Cob Products Division, Maumee, OH) and remained in these cages either individually or with their litters until euthanasia. Ground corncob nesting material is the standard bedding used at WIL Research Laboratories, LLC; therefore, for consistency with historical control data, the same bedding was used in the present study. The F_1 offspring remained with their littermates in the polyphenylsulfone cages through postnatal day (PND) 27. From PND 28 until euthanasia, the F_1 offspring were housed individually in suspended wire mesh stainless steel cages. Animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The animal facilities at WIL Research Laboratories, LLC, are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal room temperature was set to 71 ± 5°F (22 ± 3°C), the average daily relative humidity was set to 50 ± 20%, and a 12-h light cycle per day was used.

Certified Ground Rodent LabDiet 5002 was available *ad libitum* during the study. The phytoestrogen content (as total aglycone units: sum of the concentrations of genistin, genistein, daidzin, daidzein, glycitin, glycitein,

and coumestrol) of the single lot of feed used for formulating BPA diets was 312 ppm (NP Analytical Laboratories, St Louis, MO). Reverse osmosis–purified (on-site) drinking water (City of Ashland, Water Supply and Treatment, Ashland, OH) was available *ad libitum* from an automatic watering system with metal lixits in each cage.

Study Design

The study consisted of 24 female rats per dose group; 12 mated females per dose group in each of two cohorts spaced 4 weeks apart. Bred animals were assigned to the control or one of five test substance–treated groups (Table 1) using a computer program that assigned animals based on stratification of the GD 0 body weights. BPA was administered at a constant concentration (ppm) in the diet from GD 0 through lactation day (LD) 21. Table 1 summarizes the design for the F_0 animals.

Experimental Evaluations

Maternal observations. All F_0 females were allowed to deliver. The day of parturition was designated as LD 0 (dams) and PND 0 (offspring). Pups were sexed and examined for gross malformations, and the number of stillbirths and live pups was recorded. The dam and litter remained together until weaning on PND 21.

Observations for mortality and moribundity were made twice daily (A.M. and P.M.) from GD 0 through LD 21. Clinical observations were recorded daily. Twice-daily observations for dystocia (prolonged labor, delayed labor, or other difficulties) were also made during the period of expected parturition. Females were weighed on GDs 0, 3, 7, 10, 14, 17, and 20. Dams producing litters were weighed on LDs 1, 4, 7, 11, 14, 17, and 21.

Feed consumption measurements were recorded along with the body weight data. Mean test substance consumption (milligram per kilogram body weight per day) for each group was determined by multiplying the concentration of BPA in the diet (milligram BPA per gram of feed) by the gram per kilogram body weight per day feed consumption value for each animal for each interval.

Offspring (F_1) observations. Each litter was examined daily for survival. To reduce variability due to differences in litter size, litters were reduced to eight randomly selected pups of equal sex distribution, to the extent possible within each litter on PND 4. As provided in the test guidelines, litters with six pups or fewer, or that did not meet the sex ratio criteria (i.e., at least three pups per sex) were not used for neurobehavioral or neuropathological evaluation and were necropsied on PND 4. One male and one female pup from each litter that was retained were randomly assigned to one of the evaluation subsets (Table 2).

Observations for offspring mortality and moribundity were made daily from the day of parturition through euthanasia. On PNDs 4, 11, and 21 and at weekly intervals thereafter until euthanasia, each pup was removed from the home cage and received a detailed physical examination. Individual pup body weights were recorded on PNDs 1, 4, 7, 11, 14, 17, and 21 and at weekly intervals thereafter until euthanasia.

TABLE 1
Organization, Nominal Concentrations, and Target Doses

Group no.	Diet	No. of females Cohort 1	No. of females Cohort 2	Dietary concentration (ppm)	Target dose (mg/kg/day)
1	Control (basal diet)	12	12	0	0
2	BPA	12	12	0.15	0.01
3	BPA	12	12	1.5	0.1
4	BPA	12	12	75	5
5	BPA	12	12	750	50
6	BPA	12	12	2250	150

Developmental landmarks. All pups were examined daily for surface righting reflex beginning on PND 2 until attainment. Pups culled before attaining righting reflex were eliminated from statistical analysis. For this test, pups were placed in a supine position and allowed a maximum of 2 s for righting (all four paws on the surface). Failure to return to an upright position in the allotted time was considered a negative response. Female pups (one per litter) were observed daily for vaginal patency (VP) beginning on PND 25 (Adams *et al*., 1985) and continuing until VP was present, with body weight recorded on the day of attainment. Male pups (one per litter) were observed for balanopreputial separation (PPS) beginning on PND 35 (Korenbrot *et al*., 1977) and continuing until PPS was present, with body weight recorded on the day of attainment.

Detailed clinical observations (F_0 and F_1 offspring). The detailed clinical observations (DCO) were based on previously developed protocols (Gad, 1982; Haggerty, 1989; Irwin, 1968; Moser *et al*., 1988, 1991; O'Donoghue, 1989). The DCO contained observational components of a functional observational battery and involved observing the individual animal outside of the home cage in an open field for 2 min (Table 3). DCO were conducted on all F_0 females on GDs 10 and 15, on all dams on LDs 10 and 21, and on F_1 offspring on PNDs 4, 11, 21, 35, 45, and 60. Testing was performed by trained observers (without knowledge of group assignment). A separate follow-up study to evaluate F_1 pups using DCO on PNDs 4 and 11 was conducted following the present study (see Supplementary data).

Motor activity (F_1 offspring). Motor activity was measured for one male and one female per litter on PNDs 13, 17, 21, and 61 using the Kinder Scientific

TABLE 2
Offspring Allocation and Testing Schedule for Behavioral Assessments, Brain Weights, and Neuropathological/ Morphometric Evaluation

No. selected (subset)[a]	Age	Evaluation
1 pup/sex/ litter (A)[b]	PNDs 4, 11, 21, 35, 45, 60	DCO
	PNDs 13, 17, 21, 61	Motor activity
	PNDs 20, 60	Auditory startle
	PND 62	Learning and memory (Biel water maze)
	PND 72	Brain weights, neuropathological assessment, morphometry (perfused)
1 pup/sex/ litter (B)[c]	PND 22	Learning and memory (Biel water maze)
1 pup/sex/ litter (C)	PND 21	Brain weights, neuropathological assessment, morphometry (perfused)
1 pup/sex/ litter (D)	PND 21	Necropsy of all F_1 animals not selected for behavioral evaluations

[a]Of the eight pups in each litter, one male and one female pup in each litter was randomly assigned to each of the four subsets (A, B, C, or D).

[b]Subset A pups were examined for developmental landmarks. The same pups were evaluated for DCO, motor activity, auditory startle, and learning and memory (subset A).

[c]A different subset of pups was evaluated on PND 22 (subset B) than for PND 62 (subset A).

TABLE 3
Parameters for DCO

Parameters
Arousal
Backing[a]
Bizarre/stereotypic behavior
Convulsions/tremors
Ease of handling animal in hand
Ease of removal from cage
Eye color[a,b]
Eye prominence[a,b]
Fur appearance[a,b]
Gait[a]
General body posture
Grooming[a,b]
Lacrimation/chromodacryorrhea
Mobility[a]
Mucous membranes and skin (color)
Muscle tone
Palpebral closure[a,b]
Red/crusty deposits
Piloerection[a,b]
Pupillary response[a,b]
Respiratory rate/character
Salivation
Urination/defecation

[a]Not assessed for PND 4 pups due to stage of development.
[b]Not assessed for PND 11 pups due to stage of development.

Motor Monitor System (Kinder Scientific, Poway, CA). Testing was performed in a sound-attenuated room equipped with a white noise generation system set to operate at 70 ± 10 decibels (dB(A)). Each chamber consisted of a series of infrared photobeams surrounding a rectangular cage. Four-sided black enclosures surrounded the cage and decreased the potential for distraction by extraneous environmental stimuli. The calibration of each chamber was verified before each test session. The testing of treatment groups was done according to replicate sequence; no more than 24 animals were tested during a single session. Each test session was 60 min in duration; total activities (interruption of any photobeam) were recorded. These data were compiled into six 10-min subintervals for tabulation and evaluation of intrasession habituation.

Auditory startle. An auditory startle response was measured for one male and one female per litter on PNDs 20 and 60 using the Startle-Monitor System (Kinder Scientific). Testing was performed in a sound-attenuated room equipped with a white noise generation system set to operate at 70 ± 10 dB(A). Testing was performed following the DCO on PND 60. Animals were placed individually in a rectangular enclosure of appropriate size, which was enclosed in an individual isolation cabinet. Each compact cabinet measured 10.9 × 14 × 19.5 inches and was equipped with an internal light, a fan, two viewing lenses, and a complete white noise generation system. Each enclosure was equipped with a motion sensor. Each test session consisted of a 5-min acclimation period with broadband background "white" noise (65 ± 5 dB(A)). Each test session consisted of 50 trials, with a startle stimulus of 115 ± 5 dB(A) mixed-frequency "noise" burst set to 20-ms duration and 8-s intertrial interval. Startle response data were analyzed in five blocks of 10 trials each to evaluate intrasession habituation. Concurrent with the onset of the startle-eliciting stimulus, force was measured using an accelerometer attached to the bottom of each enclosure, with the output collected every millisecond for 100 ms. Startle response measurements obtained for each trial were the maximum response amplitude (MAX), measured in newtons (N), and the latency (msec) to MAX (T_{MAX}). T_{MAX} was used as a reference to verify appropriate instrument measurement of the primary startle response and was not statistically analyzed.

Biel water maze. Swimming performance and learning and memory were assessed in three phases over a 7-day period using a water-filled eight-unit T-maze similar to that described by Biel (1940). Animals were placed in the maze and were required to traverse the maze and escape by locating a platform that was hidden 2 cm beneath the surface of the water.

Phase 1 was an evaluation of swimming ability and motivation to escape from the maze and was performed in four consecutive trials on day 1 by measuring the time required for the rat to swim the length of a 4-foot straight channel. Animals were allowed 2 min to complete each trial. At the end of each trial, the animal was immediately placed at the starting position for another trial until all four trials were complete.

Phase 2 evaluated sequential learning on days 2–6. Animals were allowed two trials per day for 2 days to solve the maze in path A. Animals were then allowed two trials per day for 3 consecutive days to solve the maze in path B, which was the reverse of path A. If the animal failed to escape after 3 min, it was placed on the platform for 20 s and then removed from the maze until subsequent trials were conducted (minimum intertrial interval of 1 h).

Phase 3 (day 7) tested the animal for its memory to solve the maze when challenged in path A. The testing was conducted in two trials on 1 day as described for phase 2.

For the learning and memory phases (i.e., phases 2 and 3), the number of errors for each trial was recorded and analyzed over successive trials. An error was defined as any instance when an animal deviated from the correct channel with all four feet.

F_0 necropsy and organ weights. A gross pathological examination of all F_0 females was carried out. The maternal liver and kidneys were weighed and examined microscopically. Offspring of dams found dead or euthanized *in extremis* prior to weaning were subjected to a gross pathological examination. Offspring of a litter failing to meet the sex ratio criteria were euthanized and similarly subjected to a gross pathological examination.

F_1 offspring necropsy. A gross pathological examination of stillborn pups and pups dying between birth and PND 72 was conducted. All pups not used for neuropathological evaluation were similarly examined. Offspring that were culled on PND 4 were not examined.

Neuropathology. Pups (one per sex per litter) selected for neuropathology on PNDs 21 and 72 were perfused *in situ* with fixative (4% paraformaldehyde/1.4% glutaraldehyde), and the brain was processed for microscopic examination following at least 36 h in fixative; on PND 72, additional central nervous system (CNS) and peripheral nervous system (PNS) tissues were processed. Before processing for microscopic assessments, macroscopic evaluations including whole brain weight, as well as brain length and width, were recorded. Additionally, any abnormal coloration or lesions of the brain were recorded. Microscopic neuropathological examination was performed on one male or female per litter for a total of 10 pups per sex per dose group from all groups with the two cohorts as equally represented as possible. In addition, for PND 72 evaluations, neuropathological examinations were performed on any pup that was not selected in the original 10 pups per sex per dose group but showed potential treatment-related effects on neurobehavioral observations or measurements. The data for these additional pups were not included in the statistical analyses.

For microscopic evaluation, the CNS tissues were embedded in paraffin and PNS tissues in plastic (glycol methacrylate). Histopathological evaluation was conducted on tissues stained with hematoxylin and eosin. For animals euthanized on PNDs 21 and 72, the following brain regions were examined: olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, midbrain, cerebellum, pons, and medulla oblongata. The spinal cord was examined at cervical swellings C_3-C_7 and at lumbar swellings T_{13}-L_4 for the animals euthanized on PND 72. Also, for the animals euthanized on PND 72, the following PNS tissues were examined: cervical dorsal root fibers and ganglia (C_3-C_7); cervical ventral root fibers (C_3-C_7); eyes with retinae; lumbar dorsal root fibers and ganglia (T_{13}-L_4); trigeminal ganglia/nerves; lumbar ventral root fibers (T_{13}-L_4); optic nerves; peroneal, sciatic, sural, and tibial nerves; and skeletal muscle (gastrocnemius).

Morphometric analysis. Quantitative examinations of the brains from the selected PNDs 21 and 72 offspring were conducted using the Pax-It (MIS, Inc., Franklin Park, IL) image-capturing computer system and software (version 4.0). Specific levels analyzed were defined as follows: level 1 was a coronal section of rostral cerebrum, including caudoputamen; level 3 was a coronal section of mid-cerebrum (cerebral cortex, hippocampal formation, thalamus, etc.); and level 5 was a midsagittal section of cerebellum and pons. Levels 1, 3, and 5 corresponded to plates 11, 33, and 79, respectively, in the reference text (Paxinos and Watson, 1998). Measurements on levels 1 and 3 were bilateral, when possible, and were averaged for each animal. Measurements on level 5 were not averaged since the other halves of these tissues were sectioned transversely to enable visualization of cerebellar nuclei. Measurements were made on homologous sections to ensure that the dimensions of the regions were comparable.

Statistical Analyses

The statistical methods employed in this study were based on recent recommendations from an International Life Sciences Institute (ILSI) Expert Panel that reported on the analyses of neurodevelopmental end points in DNT studies (Holson *et al*., 2008). Pairwise comparisons, rather than trend analyses, were conducted so that potential effects at the low-dose levels would not be overlooked.

Data from nonpregnant F_0 animals were excluded from statistical analyses. With the exception of adult measurements (body weights and neurobehavioral assessments) on the F_1 generation, when a statistical analysis included measurements on multiple offspring from the same litter, the litter was used as the experimental unit and accounted for in the statistical analysis. Statistical analyses were generated from the WIL Toxicology Data Management System, or conducted in SAS version 9.1.

A number of end points were evaluated using different statistical models (Table 4). Details of the analysis models and the statistical decision-making process are included in the Supplementary data.

TABLE 4
Summary of Statistical Tests

Analysis	End points
ANOVA (1)	F_0 maternal body weight gain GDs 0–20; gestation length[a]; former implantation sites[a]; unaccounted-for sites; number of pups born[a]; live litter size[a]; DCO (continuous data)[a]
ANOVA (2)	F_1 morphometric measurements from the neocortical, hippocampal, and cerebellar areas (dependent on the outcome of the brain weight, length, and width analysis)[b]
ANOVA (3)	F_1 PPS; F_1 vaginal separation
ANOVA (4)	Preculling pup body weight gain; F_1 surface righting
ANCOVA (5)	F_0 organ weights
RANOVA (6)	F_0 maternal gestation and lactation body weights, body weight gains, and feed consumption
RANOVA (7)	F_1 adult body weights; F_1 motor activity within (total counts from six 10-min intervals) session PND 61; F_1 auditory startle response (MAX) PND 60; F_1 Biel water maze (number of errors) PND 62
RANOVA (8)	Pre- and postculling pup body weights; postculling pup body weight gain; pup motor activity within (total counts from six 10-min intervals) and across (cumulative total count) sessions PNDs 13, 17, and 21; pup auditory startle response (MAX) PND 20; pup Biel water maze (number of errors) PND 22
MANOVA (9)	F_1 brain weight, brain length, and brain width; F_1 morphometric measurements from the neocortical, hippocampal, and cerebellar areas (dependent on the outcome of the brain weight, length, and width analysis)[b]
K-W (10)	Pup viability; males per litter
Fisher (11)	DCO (scalar and descriptive data)

Note. ANCOVA, analysis of covariance; RANOVA, repeated measures ANOVA; MANOVA, multivariate ANOVA; K-W, Kruskal-Wallis test; Fisher, Fisher's exact test. Factors in the models included treatment group (TRT; 1–10), sex (2, 4, 7–9), time (6–8), cohort (blocking factor; 1–3, 5–9), and litter (random effect; 3, 4, 8). Interaction terms included TRT × Sex (2, 4, 7–9), TRT × Time (6–8), and TRT × Sex × Time (7, 8). For the ANCOVA (5), final body weight was the covariate. Individual group comparisons with the control were made by way of Dunnett's test (1–8), linear contrasts (9), or Dunn's test (10).

[a]End points analyzed using the WIL Toxicology Data Management System did not include cohort as a blocking factor.

[b]If there was a statistically significant treatment (TRT) effect for brain weight, brain length, and brain width (indicating an overall change in brain size), the analysis of microscopic examinations was conducted by MANOVA. Otherwise, the analysis of microscopic examinations was conducted by ANOVA (2).

RESULTS

Maternal (F_0) Observations

Fate, clinical observations, and reproductive end points. There were no treatment-related effects on pregnancy rates; the values were >95% in all groups. One female each in the control and 0.15-ppm groups were nongravid. In the 2250-ppm group, one female was found dead during the process of parturition on LD 0. There were no indications of the cause of death, and this single death was not attributed to BPA exposure. All other females survived to the scheduled necropsy. No treatment-related clinical findings were noted in the females at the daily examinations or during DCO at GDs 10 and 15, or LDs 10 and 21. No treatment-related effects were noted on mean gestation lengths or on parturition at any exposure level.

Gestational body weight and feed consumption. Treatment-related lower mean body weight gains ($p < 0.05$) were noted in the 750- and 2250-ppm groups during the first week of dietary exposure (GDs 0–7; Table 5). Mean body weight gains in these groups were similar to the control group during GDs 7–14 and 14–20. As a result of the initial reductions, mean body weight gains in the 750- and 2250-ppm groups were statistically significantly lower (9.5 and 22.5%, respectively) compared with the control group throughout gestation (GDs 0–20; Table 5). In addition, mean body weights were 4.7–5.2% and 8.6–10.7% lower ($p < 0.05$) than control in these same respective groups during GDs 3–20 (Fig. 1). Mean body weights and body weight gains in the 0.15-, 1.5-, and 75-ppm groups were unaffected by BPA exposure during gestation (Fig. 1, Table 5).

Mean feed consumption in the 750- and 2250-ppm groups was lower than in the control group ($p < 0.05$) and

TABLE 5
Gestational Body Weight Gain (g) for F_0 Rats Exposed to BPA

	BPA dietary concentration (ppm)					
GD	0	0.15	1.5	75	750	2250
0–7	31	34	32	31	21*	2*
7–14	38	39	40	34	36	35
14–20	78	78	79	78	75	77
0–20	147	151	151	143	133*	114*

Note. $n = 23–24$.
$*p < 0.05$.

corresponded to the lower mean body weight gain during the first week (GDs 0–7) of test diet exposure. Mean feed consumption in the 0.15-, 1.5-, and 75-ppm groups was unaffected by BPA exposure during gestation (Supplementary table 1).

Lactational body weight and feed consumption. Lactational body weight data for the dams are presented in Figure 1. As a result of the initial reductions in mean body weight gains during GDs 0–7, the mean body weights during LDs 1–17 in the 750- and 2250-ppm groups ranged from 3.8 to 5.2% and 8.1 to 9.6% lower, respectively, compared with the control group. Mean body weight gains in all groups were unaffected by BPA exposure during lactation. Mean feed consumption in the 0.15-, 1.5-, 75-, 750-, and 2250-ppm groups was unaffected by BPA exposure during lactation (Supplementary table 2).

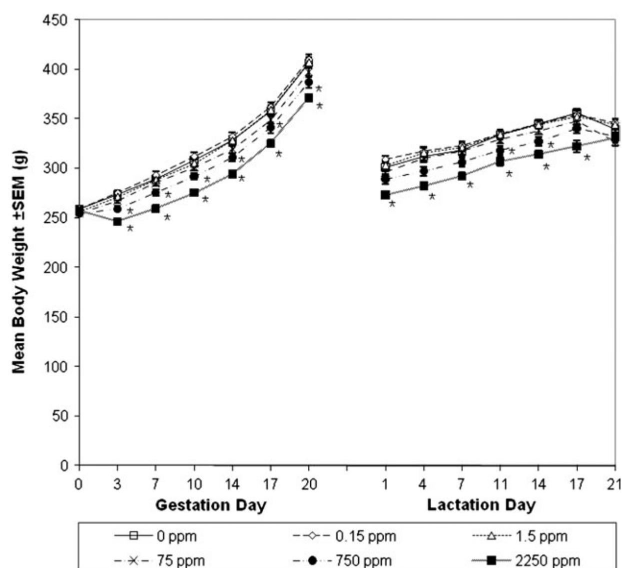

FIG. 1. F_0 female body weights during the gestational and lactational periods. Ordinate is body weights (mean gram ± SEM); abscissa is days. ''*'' indicates that the mean body weight is statistically significantly different from that of the control group using Dunnett's test ($p < 0.05$).

BPA intake. The mean doses of BPA consumed by the dams are presented in Table 6. Values during the entire lactation period for all groups were elevated as feed consumption increased to support milk production. Feed consumption is also known to increase after LD 14 due to self-feeding of the pups.

Maternal necropsy. No treatment-related gross findings or effects on the number of former implantation sites and the number of unaccounted-for sites were observed. There were no treatment-related alterations in mean kidney or liver weights in F_0 females at any exposure level when analyzed with final body weight as the covariate. There were no microscopic alterations associated with BPA exposure in the livers or kidneys of any F_0 females at any exposure level.

Offspring (F_1) Observations

F_1 fate and clinical observations. The mean number of pups born, live litter size, percentage of males per litter at birth, and postnatal survival between birth and weaning were unaffected at all BPA exposure levels (Supplementary table 3a–d). No daily clinical observations noted for F_1 pups were attributed to BPA exposure (data not shown).

F_1 body weights and developmental landmarks. Mean male and female pup body weight and preweaning body weight gain data are provided in Tables 7 and 8, respectively. Mean male and female pup body weight gains in the 750- and 2250-ppm groups were similar to the control group during PNDs 1–7, lower than in the control group ($p < 0.05$) during PNDs 7–14, and similar to the control group during PNDs 14–21. Because of the lower body weight gains during PNDs 7–14, mean male and female pup body weights in the 750-ppm group were lower ($p < 0.05$) than in the controls (4.7–5.6%) on PND 14 and/or 17 before recovering by PND 21. Mean male and female body weights in the 2250-ppm group were lower ($p < 0.05$) than in the controls (4.8–8.3%) beginning on PND 11 and continuing through the end of the preweaning period (PND 21).

Mean pup body weights and body weight changes in the 0.15-, 1.5-, and 75-ppm group males and females were unaffected by BPA exposure throughout the postnatal period.

TABLE 6
Calculated Doses of BPA

BPA dietary concentration (ppm)	Mean (range of biweekly means; mg BPA/kg/day)	
	Gestation	Lactation
0.15	0.01 (0.01–0.01)	0.03 (0.02–0.03)
1.5	0.12 (0.10–0.13)	0.25 (0.18–0.31)
75	5.85 (5.32–6.43)	13.1 (9.31–16.1)
750	56.4 (51.1–60.9)	129 (86.9–158)
2250	164 (93.8–196)	410 (288–527)

TABLE 7
Body Weight (g) of F_1 Offspring of F_0 Rats Exposed to BPA

	BPA dietary concentration (ppm)					
PND	0	0.15	1.5	75	750	2250
Males						
1	7.0	7.2	7.0	7.4	7.1	6.8
4	9.5	9.8	9.5	10.2*	9.6	9.0
4[a]	9.4	9.8	9.6	10.2*	9.7	9.0
7	15.4	16.0	15.7	16.6*	15.6	14.8
11	25.1	25.8	25.0	26.1*	24.1	23.4
14	33.2	33.7	32.9	34.0	31.6*	31.1*
17	40.6	41.6	40.5	41.6	38.7*	38.1*
21	52.2	54.3*	52.5	54.4*	50.2	49.7*
28	94	97	94	96	93	91
35	156	161	157	158	154	149
42	223	230	224	227	221	216
49	283	291	286	290	283	276
56	341	355	345	352	344	338
63	380	395	387	394	383	376
70	410	423	416	425	414	404
72	422	435	427	436	426	417
Females						
1	6.6	6.8	6.5	7.0	6.8	6.4
4	8.9	9.3	8.9	9.6*	9.2	8.4*
4[a]	9.0	9.3	8.9	9.6	9.2	8.4
7	14.7	15.2	14.7	15.4	14.8	13.9
11	24.1	24.6	23.7	24.6	23.3	22.1*
14	32.0	32.6	31.6	32.2	30.2*	29.6*
17	39.1	40.0	39.1	39.5	37.3	36.2*
21	50.2	51.7	50.0	51.1	48.1	46.8*
28	87	89	86	85	85	80
35	132	138	132	131	130	126
42	169	178	170	169	166	164
49	194	204	196	195	191	192
56	218	229	218	221	216	215
63	238	253	239	240	234	232
70	251	265	252	253	250	245
72	256	272	256	257	255	253

Note. $n = 21$–24. Statistics performed by sex through PND 21 only.
[a]After culling.
*$p < 0.05$.

TABLE 8
Preweaning Body Weight Gain (g) of F_1 Offspring of F_0 Rats Exposed to BPA

	BPA dietary concentration (ppm)					
PND	0	0.15	1.5	75	750	2250
Males						
1–4	2.5	2.6	2.6	2.8	2.5	2.1
4–7	6.0	6.1	6.2	6.5	6.0	5.8
7–14	17.7	17.7	17.2	17.4	15.9*	16.3*
14–21	19.0	20.6*	19.6	20.3*	18.7	18.6
Females						
1–4	2.3	2.5	2.3	2.7	2.4	2.0
4–7	5.8	5.9	5.9	6.0	5.7	5.5
7–14	17.2	17.5	16.8	16.8	15.4*	15.6*
14–21	18.3	19.1	18.3	18.8	17.9	17.3

Note. $n = 23$–24. Statistics performed by sex through PND 21 only. Statistics were conducted on combined sexes following weaning (PND 28 on).
*$p < 0.05$.

Statistically significant increases and decreases in body weight in the 0.15- and 75-ppm dose groups were not considered treatment related because no dose-related trends were apparent and no consistent temporal trends were observed. At 75 ppm, there were slightly fewer pups per litter at birth (mean 14.9 pups per litter vs. 15.7 pups per litter for the control group; Supplementary table 3a), and the mean gestation length was 0.3 days longer than that of the controls (Supplementary table 4); both of these factors may have contributed to the greater pup body weights in this group. There were no other statistically significant differences from the control group.

Mean postweaning offspring body weights and body weight gains were unaffected by BPA at all levels of exposure. Mean female body weight in the 2250-ppm group was slightly lower (8.0%) than in the control on PND 28 due to reduced mean body weight gain during the preweaning period. However, the difference from the control group was not statistically significant (analyzed by sexes combined), and the mean female body weights in this group were similar to the control group throughout the remainder of the postweaning period (PNDs 35–70).

There were no treatment-related effects on the age to attain surface righting response, PPS, or VP at any dose (Supplementary table 5).

Neurobehavioral assessments. No treatment-related effects were noted when DCO data were evaluated for PNDs 4, 11, 21, 35, 45, and 60. Incidental findings in the BPA-exposed groups included the following: on PND 11 only, a total of six pups (two animals from the 750-ppm group and four from the 2250-ppm group) exhibited irregular jerking movements of limbs, head, and/or body and/or jumping with all four feet in the air. These clonic movements were recorded as "convulsions" and/or "popcorn seizures." The incidence of these findings was not statistically significant (Supplementary table 6a–b) and occurred during the same period where statistically significantly reduced mean pup body weight gains were noted (PNDs 7–14) in the 750- and 2250-ppm groups (Table 8). These findings were not observed at any other age. For these six animals, there were no remarkable findings in the other end points examined as part of the detailed clinical observations. The incidence of these findings in the historical control database at PND 11 is 2 of 244 females and 0 of 243 males. Since the incidence of these findings in the present study was greater than the historical control incidence, a focused follow-up study was conducted at 2250 ppm BPA to determine reproducibility and, if appropriate, to further characterize these findings. Similar effects were noted on body weight in the

dams and offspring; however, no clonic movements were observed on PND 11 in this robust follow-up study; therefore, the initial observations were not considered to be treatment related (details of materials and methods and results in Supplementary data [table 7a–b]).

Motor activity (PNDs 13, 17, 21, and 61). Motor activity data for males and females are presented in Figure 2a–c. Motor activity was unaffected by BPA exposure at any dietary concentration. The pattern of activity over the course of the test session at each age and the overall 60-min test session values were comparable with the concurrent control (Supplementary table 8a–c).

Auditory startle (PNDs 20 and 60). Auditory startle response data are shown in Figure 3a–d. The auditory startle

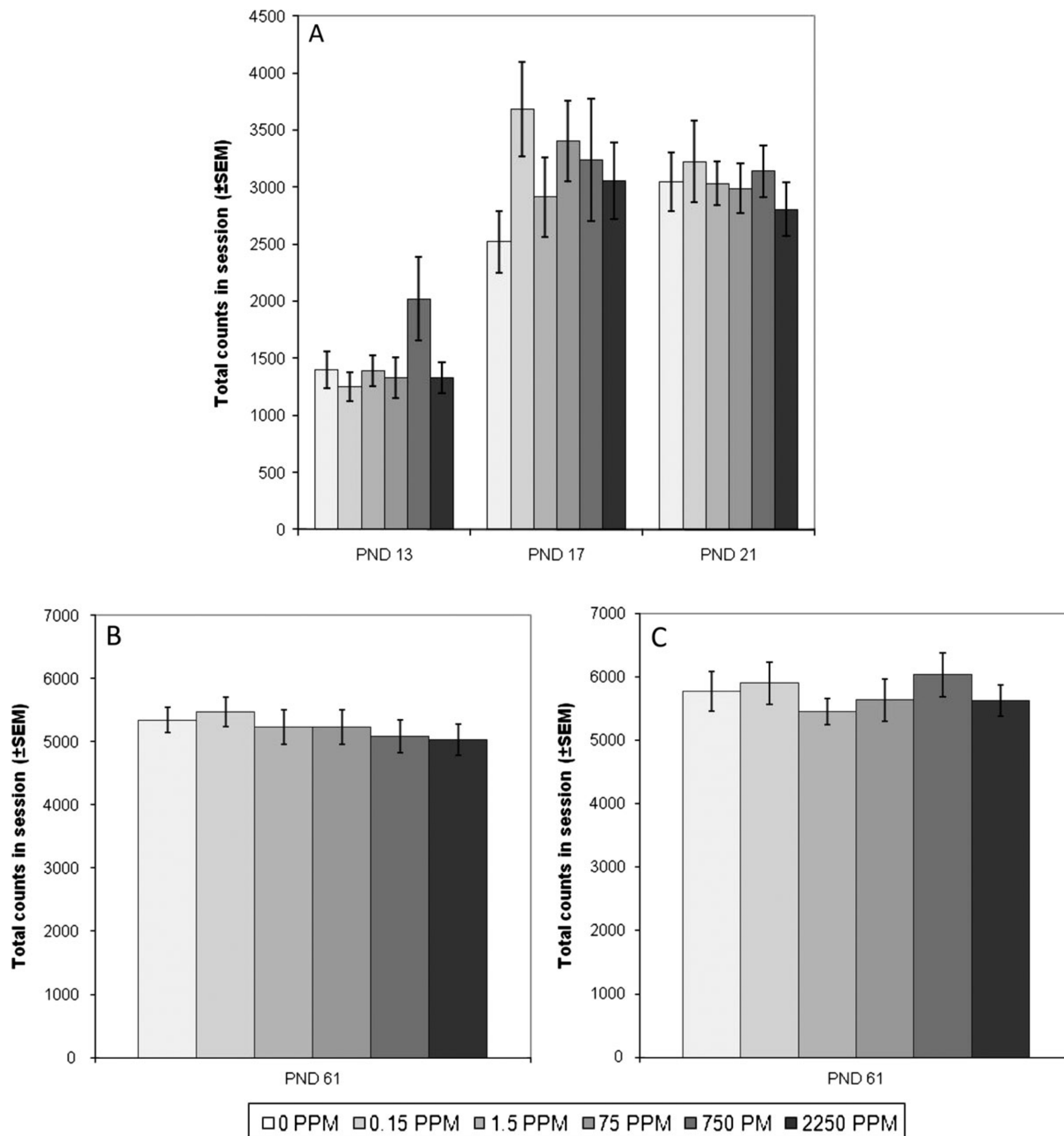

FIG. 2. Mean (±SEM) 1-h session total activity counts (ambulatory and fine movements) for F_1 offspring (combined sexes) motor activity testing on PNDs 13, 17, and 21 (A) and for F_1 males and females separately on PND 61 (B and C). There were no statistically significant effects obtained in the repeated measures ANOVA (RANOVA) for total activity counts on PNDs 13, 17, and 21, or PND 61. The ontogeny (PNDs 13, 17, and 21) data for males and females were combined, to enhance statistical power, and are presented as combined sexes because neither the treatment by sex nor the treatment by sex by time interaction was statistically significant. The data for PND 61 are presented separately for males and females because the overall RANOVA revealed a treatment by sex by time interaction.

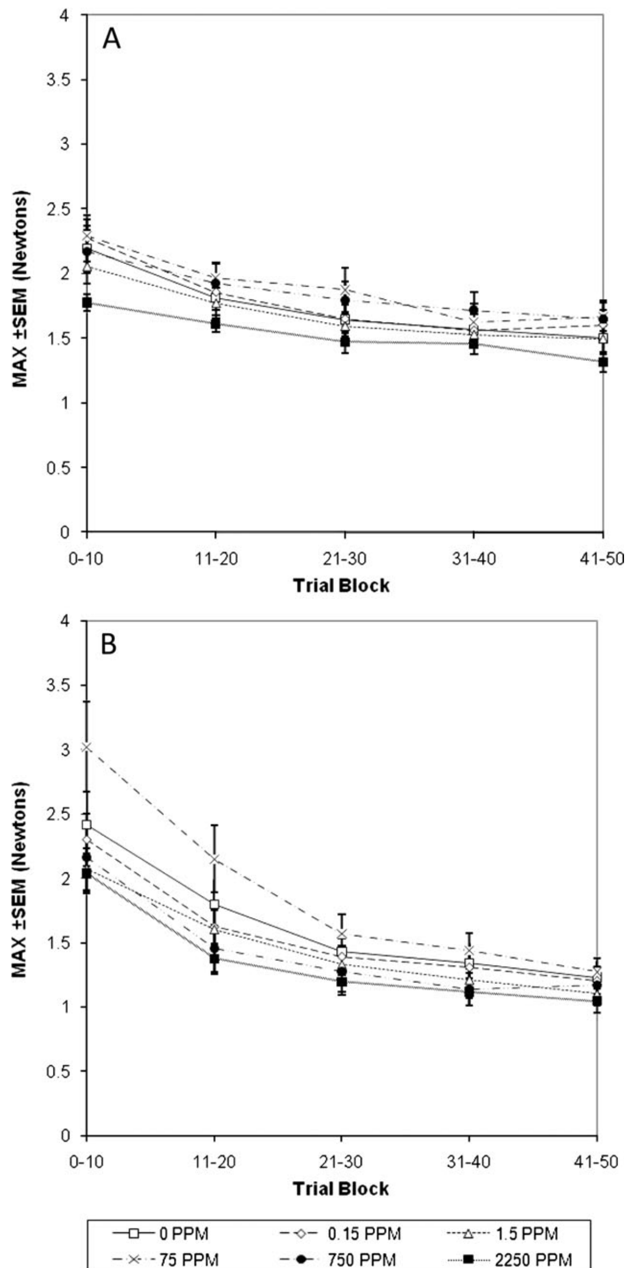

FIG. 3. Mean (±SEM) maximum startle response amplitude in newtons (MAX) across 10-trial blocks of each 50-trial testing session for F_1 offspring (combined sexes) on PND 20 (A) and PND 60 (B). There were no statistically significant effects obtained in the repeated measures ANOVA for maximum response amplitude on PND 20 or 60. The data for males and females were combined, to enhance statistical power, and are presented as combined sexes because neither the treatment by sex nor the treatment by sex by trial interaction was statistically significant.

response habituation paradigm was conducted for F_1 animals evaluated on PND 20 and for the same animals on PND 60. No effects were noted on auditory startle response amplitude or in the pattern of habituation over the 50-trial test session at either age (Supplementary table 9a–c).

Learning and memory: Biel maze swimming trials (PNDs 22 and 62). Biel maze error data are provided in Figure 4a–d. Swimming ability on day 1 of the Biel maze assessment (PND 22 or 62) was similar in all groups (Supplementary table 10a–f). There were no treatment-related effects on the number of errors committed by males or females at any dose level, at either age (Fig. 4). For females on PND 22, the overall mean number of errors committed in the 0.15- and 2250-ppm groups was lower ($p < 0.05$) than in the control group for path B (trials 5–10), which was likely due to an atypically high error rate in the PND 22 female control group in path B (i.e., outside historical control range). On PND 62, the overall mean number of errors committed by males in the 0.15-ppm group for path A (trials 1–4) was higher ($p < 0.05$) than the control group mean; however, the mean value for the control group was lower than the historical control mean values for trials 3 and 4, and performance of the 0.15-ppm males was similar to that of controls on path B. Last, the mean number of errors on PND 62 for the combined males and females in the 1.5-ppm group was higher than in the control group for trial 7 only, whereas the number of errors was similar to controls for all other trials in path B. Thus, all statistically significant differences noted were considered spurious and not BPA related because they did not occur consistently between or within testing periods, did not demonstrate any evidence of a dose-related trend, and/or were associated with atypical control responses (Supplementary table 10a–f).

F_1 offspring necropsy. No gross necropsy findings that could be attributed to BPA exposure were noted at any dose. During the postweaning period (PNDs 21–72), one female in the control group and three males in the 1.5-ppm group were found dead or euthanized *in extremis*. No remarkable gross findings were noted for any of these animals at necropsy.

Neuropathology. No treatment-related gross findings were observed in the brain at PND 21 or in the brain and spinal cord at PND 72 in F_1 animals. There were no differences from control animals with respect to treatment-related effects on brain measurements (weight, gross length or width, or microscopic measurements) on PND 21 or 72, and there were no effects on histopathological or morphometric alterations in the brain at PND 21 or 72 at any BPA exposure level for males or females (Table 9). In addition, there were no treatment-related microscopic alterations in the brain on PND 21 or in central or peripheral nervous system tissues at PND 72 at any BPA exposure level. Five pups (two males and one female in the 0.15-ppm group, one female in the 75-ppm group, and one male in the 2250-ppm group) each had a unilateral nest of embryonal cells in the lateral caudoputamen. A third male in the 0.15-ppm group had nests of embryonal cells at the lateral aspect of the caudate nucleus adjacent to the external capsule. These nests of embryonal cells most closely resembled the cells of the normal subependymal zone and occurred in the lateral caudoputamen close to the location of the lateral ventricles.

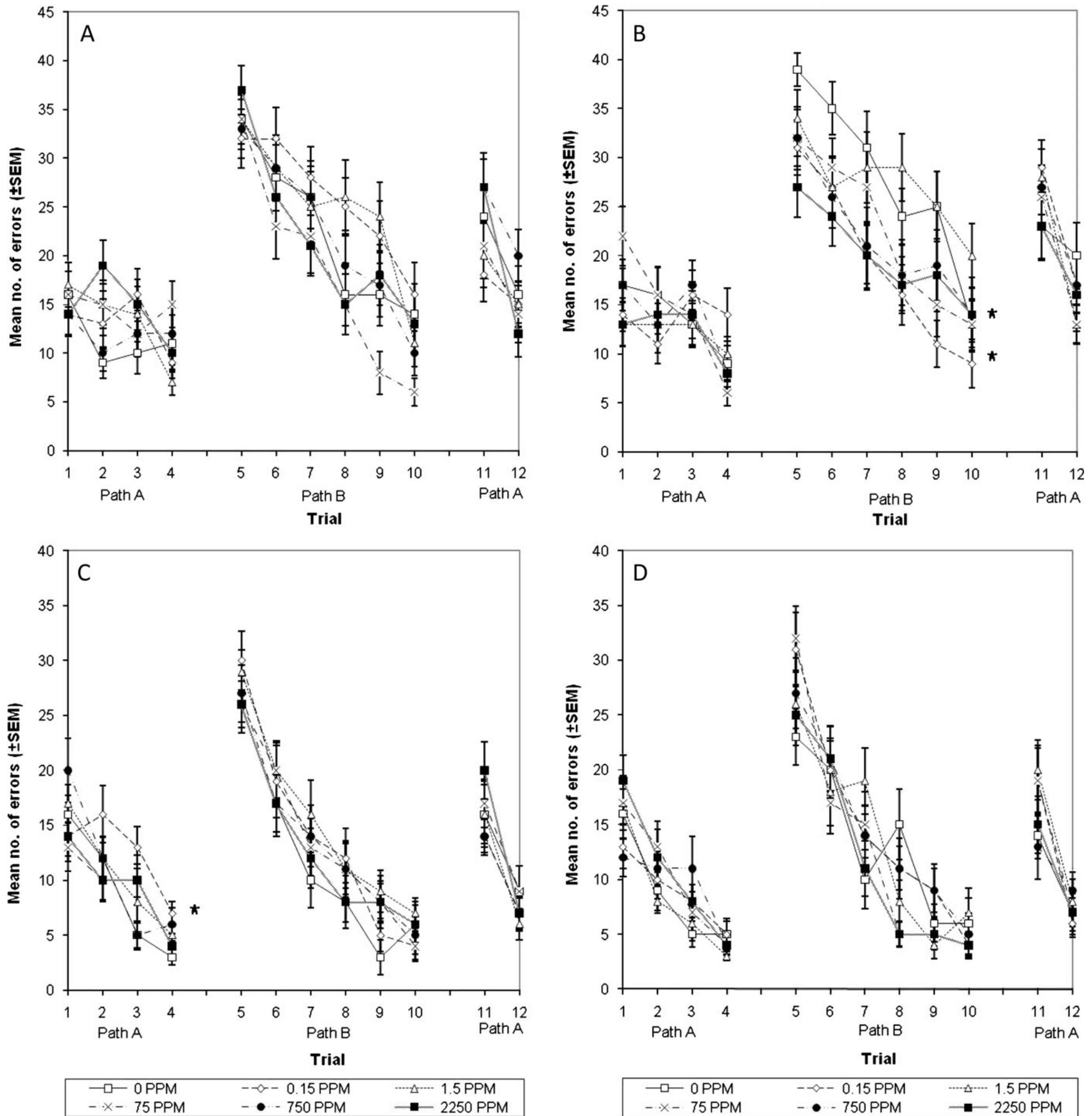

FIG. 4. Mean (±SEM) number of errors across 12 trials in the Biel water maze task for male and female F_1 offspring separately on PND 22 (males: A, females: B) and PND 62 (males: C, females: D). At PND 22, the number of errors (i.e., animal deviates from the correct channel with all four feet) in the 0.15- and 2250-ppm group females was statistically significantly lower than in the control group across the combined trials 5–10, path B (Fig. B); and at PND 62, the number of errors in the 0.15-ppm group males was statistically significantly higher than the in control group across the combined trials 1–4, path A (Fig. C). In both cases, because the treatment by trial interaction was not statistically significant, trials were not individually analyzed. When analyzed by sex, there were no additional statistically significant effects obtained in the repeated measures ANOVA (RANOVA) for mean number of errors during the 7-day learning and memory test. When the sexes were combined for analysis at PND 62, a treatment by trial interaction revealed that the mean number of errors in the 1.5-ppm group was statistically significantly higher than in the control group on trial 7 only (data not shown). The PND 22 data are presented separately for males and females because the overall RANOVA revealed a treatment-by-sex interaction (trials 5–10 only). The PND 62 data are presented separately for males and females because the overall RANOVA revealed a treatment by sex by trial interaction (trials 1–4 only). The data from males and females were combined (combined data not shown), to enhance statistical power, on PND 22 (trials 1–4 and 11–12) and on PND 62 (trials 5–10 and 11–12) because neither the treatment by sex nor the treatment by sex by trial interaction was statistically significant. Trial indicates the session trial. Trials 1–4 were conducted in the forward direction (path A), trials 5–10 were conducted in the reverse direction (path B; opposite of path A), and trials 11–12 were conducted in the forward direction (path A). Trials 1–10 tested learning and trials 11–12 probed for memory. $*p < 0.05$, Dunnett's test.

Consistent with the known process of cell migration (Paretto *et al.*, 1999), these nests were most likely migrating cells that did not complete their migration or undergo programmed cell death by PND 21. The historical control data for the laboratory indicate that "ectopic tissue" in the basal ganglia of the brain of PND 21 rats similar to the embryonal cell nests diagnosed in the present study was observed in one male and one female (of 70 and 69, respectively) control rats. Because of the occurrence of this finding in control rats of both sexes, the lack of a dose response, and unilateral nature of the finding, these observations were considered to be incidental rather than treatment related. One female in the 750-ppm group had mild cortical dysplasia that primarily involved the molecular/plexiform layer of the cerebral cortex. Nests of cells either extended up into this normally hypocellular superficial layer (within the level 1 section) or this layer was unusually expanded/focally wider within the level 4 section. The presence of this dysplasia in only one pup and lack of a dose-response relationship indicated that it was a spontaneous lesion rather than a treatment-related effect.

DISCUSSION

The potential effects of BPA on neurodevelopment following exposure of rodents during gestation and lactation have been evaluated in several studies; an assessment of these studies has indicated that there were no consistent patterns of effects across studies and end points (Chapin *et al.*, 2008). Since BPA has weak estrogen-like properties, it has been hypothesized that BPA may affect neurodevelopment via an estrogenic mode of action. A large number of studies have been reported in the published literature that have examined the developmental neurotoxicity potential of BPA. These studies have been summarized by the EU (2010). Confidence in the reliability of these studies is low because of the limitations in study design and reporting of all the available studies (EU, 2010).

The DNT test guideline was selected as a robust study design that is widely used to examine the potential of chemicals to affect neurodevelopment. Additional design elements were considered for inclusion in the study to address concerns over reported disruption of sexual differentiation of the brain and sexually dimorphic mating behaviors (e.g., lordosis). However, these end points were not selected for inclusion in the study because none has undergone the elaborate and extensive process involving multiple laboratories and reference chemicals, with ultimate consideration for relevance to human health required for validation (Balls *et al.*, 1994; Makris *et al.*, 2009; Moser, 1997). In addition, the multigeneration reproduction studies of BPA would have identified any substantive effect on mating behaviors through the alteration of some reproductive end points (e.g., time to mating, mating index, fertility index); these end points were clearly unaffected by BPA at any dose (Ema *et al.*, 2001; Tyl *et al.*, 2002, 2008d).

The present study used the Sprague-Dawley rat, which has been shown to be responsive to BPA exposure as evidenced by reduced body weight and organ weights in adults and offspring, histopathological effects in liver and kidney in adults, and delayed VP and preputial separation in offspring secondary to reduced body weights (Tyl *et al.*, 2002). The Sprague-Dawley rat has also been shown to be responsive to orally administered 17-β-estradiol (Biegel *et al.*, 1998; Tyl *et al.*, 2006). Furthermore, strain differences in response to estrogens in rats vary across tissues, so no strain can be considered more sensitive than another (Chapin *et al.*, 2008; Howdeshell *et al.*, 2008, Ryan *et al.*, 2010). Moreover, the Sprague-Dawley strain was selected for this study because it has been used extensively for DNT studies at the testing laboratory, with demonstrated sensitivity of the various tests to perturbation using appropriate positive controls.

Consideration was also given to the potential inclusion of a group treated with a steroidal estrogen in the study design for comparison with BPA-treated groups as some researchers have reported observing slight differences in motor activity measurements between sexes in rats (Tyl *et al.*, 2008a). However, these differences may depend on the device used for measurement, and even reproducibility across studies within a given laboratory has been inconsistent (Tyl *et al.*, 2008a). In addition, there are no data from studies conducted by the DNT guidelines to indicate whether the behavioral and neurodevelopmental end points in the DNT study would be sensitive to steroidal estrogens (Ferguson *et al.*, 2000), and estrogen is not among the list of positive control agents published by the ILSI Research Foundation (Crofton *et al.*, 2008). Thus, there was insufficient support from the literature to adopt the use of a steroidal estrogen as a "positive control" for the end points measured in this study. More importantly, there was no need for a concurrent positive control group as data were developed in the testing laboratory using known positive control chemicals in Sprague-Dawley rats to establish the reliability and sensitivity of the neurotoxicology test methods, under the conditions used for this study, including DCO, motor activity, auditory startle, learning and memory (Biel water maze), and morphometry.

The use of Purina Certified 5002 Rodent Diet has been challenged in studies examining estrogen-sensitive end points due to its phytoestrogen content. In the present study, the phytoestrogen level of the lot of diet used was 312 ppm in total aglycone units. The total phytoestrogen content as genistein equivalents was 290 ppm, which is below the recommended maximum of 350 ppm specified for the uterotrophic bioassay in the recently finalized OECD (2007b) Test Guideline 440. Further, this diet has been used in studies showing responses to a steroidal estrogen (17-β-estradiol; Biegel *et al.*, 1998; Tyl *et al.*, 2006, 2008b,c) and BPA (Tyl *et al.*, 2002, 2008d). Therefore, the phytoestrogen content of the diet used in this study would not be expected to influence the outcome.

TABLE 9
F_1 Male and Female Offspring Whole Brain Measurements and Morphometry

	BPA dietary concentration (ppm)					
Measurement	0	0.15	1.5	75	750	2250
Male						
PND 21						
Terminal body weight (g)	52 ± 6.2 (23)	55 ± 6.0 (23)	53 ± 5.8 (24)	55 ± 5.1 (22)	50 ± 7.9 (22)	50 ± 5.6 (23)
Relative brain weight (g/100 g body weight)	3.14 ± 0.29 (23)	3.08 ± 0.35 (23)	3.10 ± 0.23 (24)	3.02 ± 0.27 (22)	3.26 ± 0.48 (22)	3.31 ± 0.32 (23)
Brain length (mm)	18.2 ± 0.4 (23)	18.3 ± 0.4 (23)	18.2 ± 0.4 (24)	18.3 ± 0.4 (22)	18.2 ± 0.4 (22)	18.2 ± 0.4 (23)
Brain width (mm)	14.6 ± 0.3 (23)	14.8 ± 0.3 (23)	14.6 ± 0.3 (24)	14.7 ± 0.3 (22)	14.6 ± 0.4 (22)	14.7 ± 0.3 (23)
Morphometric parameters (mm)						
Level 1						
Ht of hemisphere	0.72 ± 0.03 (10)	0.73 ± 0.04 (10)	0.73 ± 0.03 (10)	0.72 ± 0.03 (10)	0.72 ± 0.02 (10)	0.71 ± 0.01 (10)
Vertical thickness of cortex	0.18 ± 0.01 (9)	0.18 ± 0.01 (10)	0.18 ± 0.02 (10)	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)
Level 3						
Radial thickness of cortex	0.16 ± 0.01 (10)	0.16 ± 0.01 (10)	0.15 ± 0.01 (10)	0.15 ± 0.01 (10)	0.15 ± 0.01 (10)	0.16 ± 0.01 (10)
Vertical ht between pyramidal neuron layers+	0.09 ± 0.01 (10)	0.09 ± 0.00 (10)	0.09 ± 0.01 (10)	0.09 ± 0.01 (10)	0.09 ± 0.01 (10)	0.09 ± 0.00 (10)
Vertical ht of dentate hilus	0.05 ± 0.01 (10)	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)
Length of ventral limb of dentate hilus	0.14 ± 0.02 (9)	0.14 ± 0.01 (9)	0.15 ± 0.01 (10)	0.14 ± 0.02 (10)	0.14 ± 0.01 (10)	0.15 ± 0.01 (10)
Level 5						
Vertical ht of cerebellum	0.48 ± 0.02 (10)	0.48 ± 0.03 (10)	0.48 ± 0.04 (10)	0.49 ± 0.03 (10)	0.48 ± 0.02 (10)	0.47 ± 0.03 (10)
Thickness of the base of cerebellar lobule 9	0.06 ± 0.00 (10)	0.06 ± 0.01 (10)	0.06 ± 0.01 (10)	0.06 ± 0.01 (10)	0.06 ± 0.00 (10)	0.06 ± 0.00 (10)
PND 72						
Terminal body weight (g)	422 ± 32.0 (23)	435 ± 40.2 (23)	427 ± 25.4 (23)	436 ± 40.1 (22)	426 ± 39.9 (21)	417 ± 44.5 (23)
Relative brain weight (g/100 g body weight)	0.52 ± 0.03 (23)	0.52 ± 0.05 (23)	0.52 ± 0.02 (23)	0.50 ± 0.04 (22)	0.51 ± 0.04 (21)	0.52 ± 0.05 (23)
Brain length (mm)	21.1 ± 0.5 (23)	21.0 ± 0.5 (23)	21.1 ± 0.4 (23)	20.9 ± 0.4 (22)	21.0 ± 0.4 (21)	21.0 ± 0.4 (23)
Brain width (mm)	15.6 ± 0.3 (23)	15.7 ± 0.3 (23)	15.6 ± 0.3 (23)	15.6 ± 0.2 (22)	15.6 ± 0.3 (21)	15.6 ± 0.4 (23)
Morphometric parameters (mm)						
Level 1						
Ht of hemisphere	0.73 ± 0.03 (10)	0.73 ± 0.04 (10)	0.71 ± 0.03 (10)	0.73 ± 0.03 (10)	0.72 ± 0.04 (10)	0.71 ± 0.03 (10)
Vertical thickness of cortex	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)
Level 3						
Radial thickness of cortex	0.17 ± 0.01 (10)	0.18 ± 0.01 (10)	0.16 ± 0.01 (10)	0.17 ± 0.01 (10)	0.17 ± 0.01 (10)	0.16 ± 0.01 (10)
Vertical ht between pyramidal neuron layers+	0.10 ± 0.00 (10)	0.10 ± 0.01 (10)	0.10 ± 0.01 (10)	0.10 ± 0.01 (10)	0.09 ± 0.01 (10)	0.10 ± 0.01 (10)
Vertical ht of dentate hilus	0.05 ± 0.00 (9)	0.05 ± 0.00 (10)	0.05 ± 0.00 (9)	0.05 ± 0.00 (9)	0.05 ± 0.00 (10)	0.05 ± 0.00 (9)
Length of ventral limb of dentate hilus	0.15 ± 0.01 (10)	0.15 ± 0.01 (10)	0.14 ± 0.01 (10)	0.14 ± 0.01 (10)	0.14 ± 0.02 (10)	0.15 ± 0.02 (10)
Level 5						
Vertical ht of cerebellum	0.53 ± 0.03 (10)	0.53 ± 0.02 (10)	0.51 ± 0.02 (10)	0.52 ± 0.03 (10)	0.51 ± 0.03 (10)	0.51 ± 0.02 (10)
Thickness of the base of cerebellar lobule 9	0.08 ± 0.01 (10)	0.07 ± 0.01 (10)	0.07 ± 0.01 (10)	0.07 ± 0.01 (10)	0.07 ± 0.01 (10)	0.08 ± 0.00 (10)
Female						
PND 21						
Terminal body weight (g)	51 ± 4.1 (23)	52 ± 5.2 (23)	51 ± 5.6 (23)	52 ± 6.9 (22)	47 ± 8.1 (22)	48 ± 5.0 (23)
Relative brain weight (g/100 g body weight)	3.07 ± 0.20 (23)	3.13 ± 0.26 (23)	3.11 ± 0.32 (23)	3.09 ± 0.40 (22)	3.36 ± 0.53 (22)	3.28 ± 0.29 (23)
Brain length (mm)	18.0 ± 0.3 (23)	18.1 ± 0.3 (23)	18.0 ± 0.4 (23)	17.8 ± 0.4 (22)	17.8 ± 0.5 (22)	17.9 ± 0.3 (23)
Brain width (mm)	14.5 ± 0.3 (23)	14.5 ± 0.3 (23)	14.5 ± 0.3 (23)	14.5 ± 0.2 (22)	14.4 ± 0.3 (22)	14.5 ± 0.3 (23)
Morphometric parameters (mm)						
Level 1						
Ht of hemisphere	0.72 ± 0.02 (10)	0.73 ± 0.02 (10)	0.71 ± 0.02 (10)	0.72 ± 0.03 (10)	0.71 ± 0.03 (9)	0.72 ± 0.02 (10)
Vertical thickness of cortex	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)	0.17 ± 0.01 (10)	0.17 ± 0.01 (10)	0.18 ± 0.01 (9)	0.17 ± 0.01 (10)

TABLE 9—*Continued*

Measurement	BPA dietary concentration (ppm)					
	0	0.15	1.5	75	750	2250
Level 3						
Radial thickness of cortex	0.16 ± 0.01 (10)	0.16 ± 0.01 (10)	0.16 ± 0.01 (10)	0.15 ± 0.01 (10)	0.15 ± 0.01 (10)	0.15 ± 0.01 (10)
Vertical ht between pyramidal neuron layers+	0.09 ± 0.01 (10)	0.09 ± 0.00 (10)	0.09 ± 0.00 (10)	0.09 ± 0.01 (10)	0.09 ± 0.01 (10)	0.09 ± 0.01 (10)
Vertical ht of dentate hilus	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)	0.05 ± 0.00 (10)
Length of ventral limb of dentate hilus	0.14 ± 0.01 (10)	0.14 ± 0.01 (10)	0.13 ± 0.02 (10)	0.13 ± 0.02 (10)	0.13 ± 0.01 (10)	0.14 ± 0.01 (10)
Level 5						
Vertical ht of cerebellum	0.48 ± 0.01 (10)	0.47 ± 0.03 (10)	0.46 ± 0.03 (9)	0.47 ± 0.03 (10)	0.46 ± 0.03 (10)	0.47 ± 0.03 (9)
Thickness of the base of cerebellar lobule 9	0.07 ± 0.00 (10)	0.06 ± 0.00 (10)	0.06 ± 0.01 (9)	0.06 ± 0.00 (10)	0.06 ± 0.00 (10)	0.06 ± 0.01 (10)
PND 72						
Terminal body weight (g)	256 ± 17.6 (22)	272 ± 19.4 (23)	256 ± 24.1 (24)	257 ± 21.4 (22)	255 ± 25.4 (22)	253 ± 22.6 (23)
Relative brain weight (g/100 g body weight)	0.79 ± 0.05 (22)	0.76 ± 0.06 (23)	0.80 ± 0.08 (24)	0.78 ± 0.07 (22)	0.78 ± 0.06 (22)	0.79 ± 0.06 (23)
Brain length (mm)	20.5 ± 0.4 (22)	20.5 ± 0.4 (23)	20.5 ± 0.5 (24)	20.3 ± 0.3 (22)	20.4 ± 0.4 (22)	20.2 ± 0.4 (23)
Brain width (mm)	15.2 ± 0.3 (22)	15.2 ± 0.3 (23)	15.1 ± 0.2 (24)	15.0 ± 0.2 (22)	15.0 ± 0.3 (22)	15.1 ± 0.3 (23)
Morphometric parameters (mm)						
Level 1						
Ht of hemisphere	0.72 ± 0.02 (10)	0.73 ± 0.03 (10)	0.73 ± 0.02 (10)	0.73 ± 0.03 (10)	0.70 ± 0.03 (10)	0.72 ± 0.04 (10)
Vertical thickness of cortex	0.18 ± 0.00 (10)	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)	0.18 ± 0.01 (10)	0.17 ± 0.01 (10)	0.18 ± 0.01 (10)
Level 3						
Radial thickness of cortex	0.17 ± 0.01 (10)	0.17 ± 0.01 (10)	0.17 ± 0.01 (10)	0.17 ± 0.01 (10)	0.17 ± 0.01 (10)	0.16 ± 0.01 (10)
Vertical ht between pyramidal neuron layers+	0.09 ± 0.00 (10)	0.10 ± 0.01 (10)	0.09 ± 0.01 (10)	0.09 ± 0.01 (10)	0.09 ± 0.01 (10)	0.09 ± 0.01 (10)
Vertical ht of dentate hilus	0.05 ± 0.00 (9)	0.05 ± 0.00 (9)	0.05 ± 0.00 (9)	0.05 ± 0.00 (10)	0.05 ± 0.00 (9)	0.05 ± 0.00 (10)
Length of ventral limb of dentate hilus	0.14 ± 0.01 (10)	0.14 ± 0.01 (10)	0.14 ± 0.02 (10)	0.14 ± 0.02 (10)	0.14 ± 0.01 (10)	0.14 ± 0.01 (10)
Level 5						
Vertical ht of cerebellum	0.50 ± 0.02 (9)	0.51 ± 0.03 (10)	0.51 ± 0.02 (10)	0.50 ± 0.02 (10)	0.49 ± 0.01 (10)	0.48 ± 0.02 (10)
Thickness of the base of cerebellar lobule 9	0.07 ± 0.00 (9)	0.07 ± 0.00 (10)	0.07 ± 0.01 (10)	0.07 ± 0.01 (10)	0.08 ± 0.00 (10)	0.07 ± 0.01 (10)

Note. Data are presented as means ± SD of 21–24 animals per group for whole brain measurements and 9–10 animals per group for morphometric parameters at each age. *n* in parentheses. When possible, all individual animal data were collected as bilateral measurements. ht, height; +, measured in the hippocampus.

The DNT study design and statistical analyses for the current study provided a robust evaluation of potential developmental neurotoxicity resulting from BPA exposure. The experimental design included multiple measures for each animal, as well as testing across time. As the DNT study design might use multiple littermates for the same test, it was important to account for the influence of genetic and maternal factors contributed by litter as well as the fact that some end points in these analyses are correlated (e.g., brain weight, length, and width). Taking these factors into consideration, the statistical methods employed in this study were based on recent recommendations from an ILSI Expert Panel for analyses of neurodevelopmental end points in DNT studies (e.g., including sex and sex by treatment interactions, litter as the unit of comparison where appropriate; Holson *et al*., 2008).

In the present study, the highest two dietary concentrations met the guideline requirements to induce systemic effects and achieve a maximum tolerated dose (750 ppm). The two lowest dietary levels (0.15 and 1.5 ppm) provided daily doses reported to produce neurobehavioral effects (reviewed by Chapin *et al*., 2008); the lowest dose approached higher estimates of adult human exposure (LaKind and Naiman, 2008). Dietary concentrations were not adjusted during lactation to offset the increased feed consumption (i.e., maintaining a more constant exposure to BPA intake throughout gestation and lactation). Since dietary concentrations were not adjusted, actual mean test substance consumption increased approximately threefold during lactation from 0.03 to 410 mg/kg/day compared with the target values of 0.01–150 mg/kg/day (Table 1). This was largely due to the greatly increased measured maternal feed consumption, and hence increased BPA intake during the last week of lactation. The increased maternal dose during lactation likely resulted in a similar increase in the dose relative to the targeted dose received by the offspring via their lactational exposure and starting of self-feeding. Hence, not adjusting the concentration in the diet provided an additional measure of

conservatism in the study design. Exposure of offspring during gestation and lactation is supported by single-dose pharmacokinetic studies in pregnant rats, which indicate that there was exposure to the test substance *in utero* at 10 or 100 mg/kg body weight (Domoradzki *et al*., 2003), as well as via the milk while nursing at 100 mg/kg body weight (Snyder *et al*., 2000). At the higher doses used in this DNT study, fetal and neonatal exposure was likely higher than in the single-dose pharmacokinetic studies due to the repeated dosing and higher maternal doses.

Treatment-related effects in the present study were limited to dose-related lower body weight and body weight gain in adults and reduced feed consumption (all beginning with the initiation of treatment), as well as lower neonatal body weight. These effects were evident in the F_0 females and F_1 males and females at 750 and 2250 ppm. F_1 pup body weight gains were lower than those of the controls during PNDs 7–14 in the 750- and 2250-ppm dose groups coincident with the increased feed consumption, and hence increased BPA intake, of the dams during the second week of lactation. No treatment-related effects were observed for any parameter at lower doses.

During the DCO of F_1 animals, clonic movements were observed in two pups at 750 ppm and in four pups at 2250 ppm on PND 11 only. The historical control data indicated that these findings have been observed in 2 of 244 females and 0 of 243 males. Since the incidence of these findings was greater than the historical control incidence, a follow-up study was conducted to determine reproducibility and, if appropriate, to further characterize these findings. The follow-up study replicated all aspects of the control and the high-dose groups of the DNT study through PND 11, but with increased sample size (two pups per sex per litter and 27 litters for the 2250-ppm dose group). No effects in the DCO, including the clonic movements observed in the DNT study on PND 11, were evident (see Supplementary data); the more definitive examination in the follow-up study provides the key evidence that the original observation of clonic movements was not treatment related. In addition, if the findings were treatment related, other observations commonly associated with treatment-related clonic movements would be expected to be seen in the DNT study. However, for the six animals showing clonic movements, there were no remarkable findings in the other end points examined as part of the detailed clinical observations or in the histopathological examination of the nervous system. Moreover, no treatment-related differences from controls for motor activity, auditory startle, or learning behavior were noted in the DNT study (which included the six animals showing clonic movements). This collective evidence, including the results from the follow-up study, supports the lack of an effect by BPA on neurodevelopment.

In conclusion, the extensive evaluations in this DNT study clearly showed that there were no functional, sensory, or cognitive deficits resulting from BPA exposure during neurodevelopment, nor was there any evidence of effects on the ontogeny of motor activity. Brain weight, length, and width, as well as histopathology and morphometry evaluations, also indicated no treatment-related effects on neurodevelopment. In addition, there were no developmental delays or evidence of effects on neurobehavioral development in this study. Since there were no neurological or neurobehavioral effects at either high or low doses of BPA, this DNT study provided evidence that there are no nonmonotonic dose responses for BPA for these end points. The NOAEL for systemic toxicity based on body weight reduction was 75 ppm (5.85 and 13.1 mg/kg/day during gestation and lactation, respectively). The NOAEL for developmental neurotoxicity of BPA was 2250 ppm (164 and 410 mg/kg/day during gestation and lactation, respectively), the highest dietary concentration tested. Based on the conditions of this study, there was no evidence that BPA is a developmental neurotoxicant in rats.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Adams, J., Buelke-Sam, J., Kimmel, C. A., Nelson, C. J., Reiter, L. W., Sobotka, T. J., Tilson, H. A., and Nelson, B. K. (1985). Collaborative behavioral teratology study: protocol design and testing procedure. *Neurobehav. Toxicol. Teratol.* **7,** 579–586.

Balls, M., Blaauboer, B. J., Fentem, J. H., Bruner, L., Combes, R. D., Ekwall, B., Fielder, R. J., Guillouzo, A., Lewis, R. W., Lovell, D. P., *et al.* (1994). Practical aspects of the validation of toxicity test procedures. *Altern. Lab. Anim.* **23,** 129–147.

Biegel, L. B., Flaws, J. A., Hirshfield, A. N., O'Connor, J. C., Elliott, G. S., Ladics, G. S., Silbergeld, E. K., Van Pelt, C. S., Hurtt, M. E., Cook, J. C., *et al.* (1998). Ninety-day feeding and one-generation reproduction study in Crl:CD BR rats with 17 ß-estradiol. *Toxicol. Sci.* **44,** 116–142.

Biel, W. C. (1940). Early age differences in maze performance in the albino rat. *J. Genetic Psychol.* **56,** 439–453.

Chapin, R. E., Adams, J., Boekelheide, K., Gray, L. E., Jr., Hayward, S. W., Lees, P. S., McIntyre, B. S., Portier, K. M., Schnorr, T. M., Selevan, S. G., *et al.* (2008). NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A. *Birth Defects Res. B Dev. Reprod. Toxicol.* **83,** 157–395.

Crofton, K. M., Foss, J. A., Hass, U., Jensen, K. F., Levin, E. D., and Parker, S. P. (2008). Undertaking positive control studies as part of developmental neurotoxicity testing: a report from the ILSI Research Foundation/Risk Science Institute expert working group on neurodevelopmental endpoints. *Neurotoxicol. Teratol.* **30,** 266–287.

Dekant, W., and Völkel, W. (2008). Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures. *Toxicol. Appl. Pharmacol.* **228,** 114–134.

Domoradzki, J. Y., Pottenger, L. H., Thornton, C. M., Hansen, S. C., Card, T. L., Markham, D. A., Dryzga, M. D., Shiotsuka, R. N., and Waechter, J. M., Jr. (2003). Metabolism and pharmacokinetics of bisphenol A (BPA) and the embryo-fetal distribution of BPA and BPA-monoglucuronide in CD Sprague-Dawley rats at three gestational stages. *Toxicol. Sci.* **76,** 21–34.

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

European Food Safety Authority (EFSA). (2006). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to 2,2-bis(4-hydroxyphenyl)propane. *EFSA J.* **428,** 1–75.

European Union (EU). (2010). *European Union Risk Assessment Report: 4,4'-Isopropylidenediphenol (Bisphenol-A).* Available at: http://ecb.jrc.ec.europa.eu/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/ADDENDUM/bisphenola_add_325.pdf. Accessed February 22, 2010.

Ferguson, S. A., Scallet, A. C., Flynn, K. M., Meredith, J. M., and Schwetz, B. A. (2000). Developmental neurotoxicity of endocrine disruptors: focus on estrogens. *Neurotoxicology.* **21,** 947–956.

Food and Drug Administration (FDA). (2008). *Draft Assessment of Bisphenol A for Use in Food Contact Applications* Available at: http://www.fda.gov. Accessed August 14, 2008.

Gad, S. C. (1982). A neuromuscular screen for use in industrial toxicology. *J. Toxicol. Environ. Health* **9,** 691–704.

Haggerty, G. C. (1989). Development of tier I neurobehavioral testing capabilities for incorporation into pivotal rodent safety assessment studies. *J. Am. Coll. Toxicol.* **8,** 53–69.

Health Canada. (2008). *Screening Assessment for the Challenge, Phenol, 4,4'–(1-methylethylidene)bis (Bisphenol A), Chemical Abstracts Service Registry Number 80-05-7, Environment Canada, Health Canada. October 18, 2008.* Available at: http://www.ec.gc.ca/substances/ese/eng/challenge/batch2/batch2_80-05-7_en.pdf. Accessed February 22, 2010.

Holson, R., Freshwater, L., Maurissen, P. J., Moser, V. C., and Phang, W. (2008). Statistical issues and techniques appropriate for developmental neurotoxicity testing: a report from the ILSI Research Foundation/Risk Science Institute expert working group on neurodevelopmental endpoints. *Neurotoxicol. Teratol.* **30,** 326–348.

Howdeshell, K. L., Furr, J., Lambright, C. R., Wilson, V. S., Ryan, B. C., and Gray, L. E., Jr. (2008). Gestational and lactational exposure to ethinyl estradiol, but not bisphenol A, decreases androgen-dependent reproductive organ weights and epididymal sperm abundance in the male Long Evans hooded rat. *Toxicol. Sci.* **102,** 371–382.

Irwin, S. C. (1968). Comprehensive observational assessment: Ia. A systematic quantitative procedure for assessing the behavioral physiological state of the mouse. *Psychopharmacologia.* **13,** 222–256.

Korenbrot, C. C., Huhtaniemi, I. T., and Weiner, R. I. (1977). Preputial separation as an external sign of pubertal development in the male rat. *Biol. Reprod.* **17,** 298–303.

LaKind, J. S., and Naiman, D. Q. (2008). Bisphenol A (BPA) daily intakes in the United States: estimates from the 2003–2004 NHANES urinary BPA data. *J. Expo. Sci. Environ. Epidemiol.* **18,** 608–615.

Makris, S. L., Raffaele, K., Allen, S., Bowers, W. J., Hass, U., Alleva, E., Calamandrei, G., Sheets, L., Amcoff, P., Delrue, N., *et al.* (2009). A retrospective performance assessment of the developmental neurotoxicity study in support of OECD Test Guideline 426. *Environ. Health Perspect.* **117,** 17–25.

Moser, V. C. (1997). Neurobehavioral screening methods. *Neurotoxicology.* **18,** 925–1101.

Moser, V. C., McCormick, J. P., Creason, J. P., and MacPhail, R. C. (1988). Comparison of chlordimeform and carbaryl using a functional observational battery. *Fundam. Appl. Toxicol* **11,** 189–206.

Moser, V. C., McDaniel, K. L., and Phillips, P. M. (1991). Rat strain and stock comparisons using a functional observational battery: baseline values and effects of Amitraz. *Toxicol. Appl. Pharmacol.* **108,** 267–283.

National Research Council. (1996). *Guide for the Care and Use of Laboratory Animals.* Institute of Laboratory Animal Resources, Commission on Life Sciences; National Academy Press, Washington, DC.

O'Donoghue, J. L. (1989). Screening for neurotoxicity using a neurologically based examination and neuropathology. *J. Am. Coll. Toxicol.* **8,** 97–115.

Organization for Economic Cooperation and Development (OECD). (1998). *OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1, OECD Principles of Good Laboratory Practice.* OECD, Environment Directorate, Paris, France. Available at: http://www.olis.oecd.org/olis/1998doc.nsf/LinkTo/env-mc-chem(98)17. Accessed February 22, 2010.

Organization for Economic Cooperation and Development (OECD). (2002). *OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 13. The Application of the OECD Principles of GLP to the Organization and Management of Multi-Site Studies.* OECD Environment Directorate, Paris, France. Available at: http://www.olis.oecd.org/olis/2002doc.nsf/LinkTo/env-jm-mono(2002)9. Accessed February 22, 2010.

Organization for Economic Cooperation and Development (OECD). (2007a). *OECD Guidelines for the Testing of Chemicals. Test No. 426: Developmental Neurotoxicity Study.* OECD Environment Directorate, Paris, France. Available at: http://oberon.sourceoecd.org/vl=692122/cl=21/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s26/p1. Accessed February 22, 2010.

Organization for Economic Cooperation and Development (OECD). (2007b). *OECD Guidelines for the testing of Chemicals. Test No. 440: Uterotrophic Bioassay in Rodents: A short-term screening test for oestrogenic properties.* OECD Environment Directorate, Paris, France. Available at: http://oberon.sourceoecd.org/vl=692122/cl=21/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s34/p1. Accessed February 22, 2010.

Paretto, P., Merighi, A., Fasolo, A., and Bonfanti, L. (1999). The subependymal layer in rodents: a site of structural plasticity and cell migration in the adult mammalian brain. *Brain Res. Bull.* **49,** 221–243.

Paxinos, G., and Watson, C. (1998). In *The Rat Brain in Stereotaxic Coordinates*, 4th ed. Academic Press, New York.

Ryan, B. C., Hotchkiss, A. K., Crofton, K. M., and Gray, L. E., Jr. (2010). In utero and lactational exposure to Bisphenol A, in contrast to ethinyl estradiol, does not alter sexually dimorphic behavior, puberty, fertility and anatomy of female LE rats. *Toxicol. Sci.* **114,** 133–148.

Snyder, R. W., Maness, S. C., Gaido, K. W., Welsch, F., Sumner, S. C., and Fennell, T. R. (2000). Metabolism and disposition of bisphenol A in female rats. *Toxicol. Appl. Pharmacol.* **168,** 225–234.

Tyl, R. W., Crofton, K., Moretto, A., Moser, V., Sheets, L. P., and Sobotka, T. J. (2008a). Identification and interpretation of developmental neurotoxicity effects: a report from the ILSI Research Foundation/Risk Science Institute expert working group on neurodevelopmental endpoints. *Neurotoxicol. Teratol.* **30,** 349–381.

Tyl, R. W., Myers, C. B., Marr, M. C., Castillo, N. P., Seely, J. C., Sloan, C. S., Veselica, M. M., Joiner, R. L., Van Miller, J. P., and Simon, G. S. (2006). Three-generation evaluation of dietary para-nonylphenol in CD (Sprague-Dawley) rats. *Toxicol. Sci.* **92,** 295–310.

Tyl, R. W., Myers, C. B., Marr, M. C., Castillo, N. P., Veselica, M. M., Joiner, R. L., Dimond, S. S., Van Miller, J. P., and Hentges, S. G. (2008b). One-generation reproductive toxicity study of dietary 17ß-Estradiol (E2; CAS No. 50-28-2) in CD-1® (Swiss) mice. *Reprod. Toxicol.* **25,** 144–160.

Tyl, R. W., Myers, C. B., Marr, M. C., Sloan, C. S., Castillo, N., Veselica, M. M., Seely, J. C., Dimond, S. S., Van Miller, J. P., Shiotsuka, R. S., *et al*. (2008c). Two-generation reproductive toxicity evaluation of dietary 17ß-estradiol (E2; CAS No. 50-28-2) in CD-1® (Swiss) mice. *Toxicol. Sci.* **102,** 392–412.

Tyl, R. W., Myers, C. B., Marr, M. C., Sloan, C. S., Castillo, N., Veselica, M. M., Seely, J. C., Dimond, S. S., Van Miller, J. P., Shiotsuka, R. S., *et al*. (2008d). Two-generation reproductive toxicity study of dietary bisphenol A (BPA) in CD-1® (Swiss) mice. *Toxicol. Sci.* **104,** 362–384.

Tyl, R. W., Myers, C. B., Marr, M. C., Thomas, B. F., Keimowitz, A. R., Brine, D. R., Veselica, M. M., Fail, P. A., Chang, T. Y., Seely, J. C., *et al*. (2002). Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. *Toxicol. Sci.* **68,** 121–146.

U.S. Environmental Protection Agency (EPA). (1989). *Good Laboratory Practice Standards (*40 CFR Part 792*)*, 18 September 1989. EPA, Washington, DC. Available at: http://www.epa.gov/lawsregs/search/40cfr.html. Accessed February 22, 2010.

U.S. Environmental Protection Agency (EPA). (1998). *Health Effects Test Guidelines: OPPTS 870.6300, Developmental Neurotoxicity Study*. EPA 712-C-98–239. August 1998. EPA, Washington, DC. Available at: http://www.epa.gov/oppts/pubs/frs/publications/Test_Guidelines/series870.htm. Accessed February 22, 2010.