

Gestational Exposure to Bisphenol A Produces Transgenerational Changes in Behaviors and Gene Expression

Jennifer T. Wolstenholme, Michelle Edwards, Savera R. J. Shetty, Jessica D. Gatewood, Julia A. Taylor, Emilie F. Rissman,* and Jessica J. Connelly

Departments of Biochemistry and Molecular Genetics (J.T.W., M.E., S.R.J.S., J.D.G., E.F.R.) and Cardiovascular Medicine (J.J.C.), University of Virginia School of Medicine, Charlottesville, Virginia 22908; and Division of Biological Sciences (J.A.T.), University of Missouri, Columbia, Missouri 65211

Bisphenol A (BPA) is a plasticizer and an endocrine-disrupting chemical. It is present in a variety of products used daily including food containers, paper, and dental sealants and is now widely detected in human urine and blood. Exposure to BPA during development may affect brain organization and behavior, perhaps as a consequence of its actions as a steroid hormone agonist/antagonist and/or an epigenetic modifier. Here we show that BPA produces transgenerational alterations in genes and behavior. Female mice received phytoestrogen-free chow with or without BPA before mating and throughout gestation. Plasma levels of BPA in supplemented dams were in a range similar to those measured in humans. Juveniles in the first generation exposed to BPA *in utero* displayed fewer social interactions as compared with control mice, whereas in later generations (F₂ and F₄), the effect of BPA was to increase these social interactions. Brains from embryos (embryonic d 18.5) exposed to BPA had lower gene transcript levels for several estrogen receptors, oxytocin, and vasopressin as compared with controls; decreased vasopressin mRNA persisted into the F₄ generation, at which time oxytocin was also reduced but only in males. Thus, exposure to a low dose of BPA, only during gestation, has immediate and long-lasting, transgenerational effects on mRNA in brain and social behaviors. Heritable effects of an endocrine-disrupting chemical have implications for complex neurological diseases and highlight the importance of considering gene-environment interactions in the etiology of complex disease. (*Endocrinology* 153: 3828–3838, 2012)

During normal brain development, steroid hormones, particularly estrogens and androgens, play a role in sculpting the neural circuits that underlie a variety of behaviors (1, 2). These hormones, in turn, regulate transcription of many other genes including vasopressin (*Avp*) and oxytocin (*Oxt*), which are also important for expression of social behaviors (3, 4). Thus, it is not a surprise that endocrine-disrupting chemicals (EDC) can have similar effects and influence organization of the developing brain (5, 6), which can influence expression of species-typical social behaviors (7–9). In addition to the behavioral effects of EDC, it is believed that EDC can have epigenetic actions

(7, 8), and some transgenerational effects have been found (10–13). One EDC, vinclozolin, has been reported to have effects on behavior (14–16); unfortunately, the doses used in these studies are higher than typical human exposures. We, and others, have shown that gestational exposure to another EDC, bisphenol A (BPA), influences the display of juvenile social behaviors (17, 18) and can alter DNA methylation (19–22). In addition, BPA has transgenerational effects on sperm fertility and other reproductive parameters in male rats (23). We undertook this study to characterize the effects of BPA on social behaviors at a dose commonly found within the human population and de-

ISSN Print 0013-7227 ISSN Online 1945-7170

Printed in U.S.A.

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doi: 10.1210/en.2012-1195 Received February 20, 2012. Accepted May 7, 2012.

First Published Online June 15, 2012

* E.R., corresponding author.

Abbreviations: BPA, Bisphenol A; C_t, cycle threshold; EDC, endocrine-disrupting chemicals; E18.5, embryonic d 18.5; LC, liquid chromatography; MS, mass spectrometry; PN21, post-natal d 21; qPCR, quantitative real-time PCR.

termine whether these effects are transmitted to unexposed future generations.

BPA is a man-made compound used to manufacture polycarbonate plastics, epoxy resins, and other commonly used items. Human exposure to BPA is widespread, and it has been detected in urine in over 90% of all humans sampled (24). Public health concerns have been fueled by findings that BPA exposure can influence brain development and some behaviors (7–9). For example, *in vivo* perinatal exposure to BPA in rodents modifies sex differences in the brain (25–27). In mice, prenatal exposure to BPA is associated with increased anxiety, aggression, cognitive impairments, and decreased novelty seeking (6, 17, 28–36). In the offspring of BPA-exposed monkeys, males displayed fewer social behaviors and were more exploratory (37). In humans, BPA exposure during gestation has been associated with hyperactivity and aggression in 2-yr-old children (38) and with anxiety and depression in older children (39). Together, these reports and many others demonstrate that BPA exposure during gestation affects several types of behaviors in a number of species.

In the present studies, we used a dose of BPA, incorporated into food, that yielded concentrations of unconjugated BPA in maternal plasma typical of those found in human blood. We assessed behaviors in juveniles in the F₁ offspring of treated dams and then conducted brother-sister mating through to the F₄ offspring. We tested F₂ and F₄ offspring for the same juvenile behaviors. We conducted a global gene expression study using embryonic [embryonic d 18.5 (E18.5)] whole brains and followed up with quantitative real-time PCR (qPCR) to assess gene expression for candidate genes involved in social behaviors. We chose to use embryonic brains because postbirth maternal behaviors might have affected gene transcription (40). Also, by using embryos, we were able to select animals to assay based on their interuterine positions. We found that gestational exposure to BPA affected expression of a number of genes in F₁ brains, and some of these changes persisted into the F₄ generation. To our knowledge, our findings are the first to demonstrate transgenerational actions of BPA on juvenile behavior and on neural gene expression.

Materials and Methods

Animals

All procedures were conducted in compliance with the University of Virginia Animal Use and Care Committee. All mice were housed on a 12-h light, 12-h dark cycle (lights off at 1300 h). Adult female C57BL/6J (B6) mice (n = 5 per group) were randomly assigned to either a phytoestrogen-free chow (Harlan Teklad, Madison, WI; TD95092) or the same chow supplemented

with 5 mg BPA/kg diet (Harlan Teklad; TD09386). Mice started consuming these diets 7–10 d before pairing with a male. Males were removed after 1 wk and were not present when pups were delivered. All females consumed their assigned diets and water *ad libitum*. Over the last 10 d of gestation, dams ingested about 4 g daily of this type of chow (17). At this dose of BPA in chow, we calculated intake to be roughly 20 µg BPA daily.

For embryo collection, females were checked daily for copulatory plugs. The day a plug was discovered was designated E0.5. Pregnant females were collected in the morning on E18.5, rapidly killed with isoflurane, and embryos extracted. Embryo brains were collected quickly and frozen on dry ice. Embryos were sexed by PCR (41), and we limited our use to embryos positioned *in utero* next to only one male littermate to reduce any potential variation caused by intrauterine position (42). We collected brains from six litters from the F₁ generation (control litters n = 3, BPA litters n = 3) and eight litters from the F₄ generation (control litters n = 4, BPA litters n = 4).

For the behavioral studies, within 12 h after birth, all pups from control and BPA-consuming dams were fostered to a dam on control diet that had given birth within the past 24 h. We did this to limit offspring exposure to BPA to the gestational period and because differences in maternal behavior caused by BPA might affect offspring behavior (17). Foster dams (n = 10) had mixed litters of their biological, same-age pups (not included in the study) and fostered pups. For identification purposes, we randomly clipped tail tips of either the biological (control litters n = 3, BPA litters n = 4) or foster pups at the time of fostering. All pups (control n = 12 males and n = 10 females, BPA n = 18 males and 11 females) remained with their foster dams until postnatal d 21 (PN21), at which time they were placed on standard rodent chow containing phytoestrogens (Harlan Teklad diet 7912), group housed by litter and sex, and tested for behaviors (see below). F₁ brother-sister pairs (BPA n = 9, control n = 8) were used to produce the transgenerational lines. At the time of breeding and throughout rearing, the mice consumed standard rodent chow, and none of the subsequent offspring were fostered.

BPA assay

Pregnant dams consuming control (n = 3) or BPA-supplemented (n = 6) diet were deeply anesthetized on E18.5 with isoflurane, and blood was collected by cardiac puncture. Pooled sets of serum samples (~0.3–0.8 ml) were dispensed into glass tubes, spiked with an internal standard, and extracted twice with methyl *tert*-butyl ether. The ether extract was dried in glass tubes under nitrogen and reconstituted in 60:40 methanol/water. Unconjugated (free) BPA was measured using isotope dilution liquid chromatography (LC)/mass spectrometry (MS) with [¹³C]BPA (Cambridge Isotopes Laboratories, Waltham, MA) as the internal standard. Serum BPA was quantified by LC/MS using a Thermo Finnigan Surveyor MSQ plus connected to an integrated Thermo Accela LC system; analytes were detected using electrospray ionization with negative polarity, a cone voltage of 70V, and probe temperature of 600 C. Separations were performed on a 1.9-µm Hypersil Gold HPLC column (50 × 2.1 mm) with a mobile phase gradient running from 20–95% acetonitrile over 6 min at 550 µl/min. Thermo Xcalibur software was used to autotune, acquire, and process the LC/MS data. Quantification was made against standard curves of the analytes. The limit of quantification (43) for BPA in serum by these methods was 0.5 ng/ml based on extraction of 0.5

ml serum, and reported values below this level were estimated by extrapolation of the standard curve to zero. Extraction efficiency was assessed from recovery of the internal standard and averaged over 95%. BPA was not detected by HPLC in either the assay blanks or in the solvent blanks used in the standard curve or in the pipettes used to collect the plasma or tubes used for storage. Solvents and water used in the assay were HPLC grade and previously tested negative for BPA.

Behavior tests

All habituation and behavior tests were conducted in the dark (between 1300 and 1800 h) under red light. Behaviors were recorded and later scored by an observer blind to sex and treatment group. Each apparatus was cleaned with 10% ethanol and wiped dry between tests.

Juvenile social interactions

On PN20, the day before weaning, mice were singly housed in a novel standard mouse cage, with bedding but no food or water, for 1 h to habituate to this novel environment. After habituation, mice were returned to their home cages with their siblings and dam. On PN21, mice were again habituated to the test room in a clean standard mouse cage for 1 h, and then a same age, sex, and prenatally treated mouse from another litter was added to the cage for the test. Social interactions were recorded for both mice for 10 min. Mice were evaluated for a number of social and nonsocial behaviors using Noldus Observer (version 5.0) software (Noldus, Leesburg, VA). Testing details are published (44, 45). Briefly, individual behaviors were categorized into four groups: social, nonsocial, investigative, and play soliciting. Social and nonsocial behaviors were scored for the duration of time spent engaging in each behavior. Investigative and play-soliciting behaviors were scored for the frequency of each behavior performed. The social behavior categories included side-by-side sitting, grooming their partners, and side-by-side interactions. Nonsocial behaviors consisted of exploring, self-grooming, and sitting alone. The frequency of investigative behaviors included acts of nose-to-nose or anogenital sniffing, digging, and jumping. Play-soliciting behaviors were crawling over or under the other mouse, pushing the other mouse, approaching the other mouse head-on, and following the other mouse. After these interactions, mice were housed singly for the duration of the behavioral testing schedule.

Elevated plus maze

On PN22, each mouse was tested on the elevated plus maze as previously described (46). Behavior was recorded for 10 min. The total time spent in the closed and open arms and the numbers of crosses through the center were scored. A complete zone entry was defined as all four paws in the zone. Time spent in the center of the maze was calculated based on the total duration of the test less the time in the two arms. The open arm was subdivided into proximal and distal halves, and time in each was recorded.

Social preference tests

On PN24, male and female mice from each test group were habituated to a testing room for 1 h and then placed into the center section of a three-chambered Plexiglas box (76.2 × 26.67 × 17.78 cm), divided by black Plexiglas walls and backed by black Plexiglas so that the center section was darkened on

three sides with two openings leading to the outer chambers, each containing a small metal cylinder with a round top (10.16 cm diameter × 13.97 cm) and vertical bars (spaced 1 cm apart). Mice were habituated to the test box in the center section with both doors closed for 10 min. After 10 min, the doors were opened and the mouse was allowed to freely explore all three chambers for an additional 10 min. Mice were once again closed into the center section, and a novel adult male mouse was randomly placed in one of the two cylinders; the other cylinder remained empty. The doors were opened to allow mice to explore all three chambers, and data were collected for 10 min. The time spent in each chamber, the time spent sniffing each cylinder, and the numbers of entries into each side were all scored by an observer blind to sex and treatment group (adapted from Refs. 47 and 48 and described in detail in Ref. 17). Entry in each chamber was defined when all four paws entered the chamber.

Microarray analysis

Total RNA was isolated from brain tissue of BPA or control exposed male and female F₁ generation embryos at d 18.5 (n = 3 per group). E18.5 was chosen as the time point for gene expression changes because BPA is still present in the maternal-fetal circulation, as opposed to after the pups are born. Additionally, maternal behavior can influence DNA methylation (40), and BPA may affect the dams' interactions with their pups (17, 49, 50). Thus, to avoid these potential confounds, we assayed embryonic brain. As an initial study, we chose to measure transcription profiles from whole brain.

RNA quality and quantity was assessed using an Agilent (Roseville, CA) bioanalyzer and was labeled using the TotalPrep RNA Labeling Kit (Ambion, Austin, TX). All RNA samples had 260:280 ratios greater than 1.9, indicating high-quality RNA. Three biological samples from each treatment group were hybridized to whole-genome Illumina MouseWG-6 version 2.0 Expression Bead Chips (San Diego, CA) (n = 12 arrays), and data were extracted using an Illumina Beadstation and iScan system. Quality control analyses were performed on the array data by examining intensity histogram plots, chip-chip linearity and inter-chip correlations of intensity values. Samples were quantile normalized using the BeadArray package (51) in the Bioconductor software suite. To determine differential gene expression affected by diet and/or sex, we performed a two-way ANOVA on the dataset using the TIGR Multiple expression viewer (T-Mev) (52). *P* values were corrected for multiple comparisons using the *q* value program in Bioconductor (53). Because only five transcripts in any comparison showed significance after correcting for multiple comparisons, we display the top genes significant at uncorrected *P* < 0.01.

Quantitative real-time PCR

To confirm the microarray findings and since BPA has known effects on candidate genes not identified by our microarray analysis within the current model, total RNA was isolated from the brain tissue of male and female embryos at d 18.5 from the F₁ (n = 3 per group) and F₄ generation (n = 5–6 per group) from dams given control or BPA-supplemented diet. We hypothesized that if BPA acted via one or more of the estrogen receptors, it might up- or down-regulate the receptor and its targets, perhaps in a manner similar to the natural ligand. cDNA was generated from 500 ng total RNA by reverse transcription with iScript

cDNA kit (Bio-Rad, Hercules, CA). Real-time PCR was performed using the iCycler iQ System (Bio-Rad) for TaqMan- and SYBR Green-based detection. Biological replicate samples were run in quadruplicate on either one or two plates. The average of the four replicates was used for data analysis. We were particularly interested in assessing the levels of known BPA targets such as estrogen receptor α (*Esr1*), estrogen receptor β (*Esr2*), the membrane-bound estrogen receptor (*Gper*, previously called *Gpr30*), and the estrogen-related receptor γ (*Esrrg*). Considering the fact that gestational BPA exposure altered social behaviors, we also assayed known estrogen target genes involved in social behaviors such as oxytocin (*Oxt*), vasopressin (*Avp*), and their receptors oxytocin receptor (*Oxtr*) and vasopressin receptor 1a (*Avpr1a*), the primary vasopressin receptor within the brain. TaqMan probes for *Esr1*, *Esr2*, *Gper*, *Esrrg*, *Oxtr*, *Oxt*, *Avpr1a*, *Avp*, and *B2M* (β 2-microglobulin, control gene) were obtained from Applied Biosystems (Carlsbad, CA). Previously, we found that gestational BPA exposure (using a lower dose) altered expression of DNA methyltransferase genes (*Dnmt*) and the glutamate transporter *Slc1a1* (18). In our microarray dataset, one gene was significantly affected by diet, caspase 9 (*Casp9*). To assess transcript levels of these genes, SYBR Green primers were designed for *Casp9*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Slc1a1*, and *Ppib* (peptidylprolyl isomerase B, control gene) as previously described (18). Quantification of candidate gene expression levels was calculated based on the threshold cycle (C_t) for each well using the provided software and normalized to *B2M* for TaqMan assays and *Ppib* for SYBR Green assays as endogenous controls. All data are normalized to the control male group.

Statistical analysis

All data were analyzed using NCSS (2001). For gene expression data, normalized gene expression was calculated using the $\Delta\Delta C_t$ method (54) and is relative to the control male group. For data analysis, we used two-way ANOVA with sex and diet as factors. Significant results were assessed by Fisher's exact *post hoc* tests that adjust significance levels to take multiple comparisons into account.

Results

BPA exposure levels

To ensure our dose of BPA was relevant to humans, we assayed blood levels in dams on gestational d 18.5, comparing dams ingesting control diet with those consuming BPA (5 mg/kg diet). Normal blood levels of free BPA in pregnant women summarized from three separate cohorts report a median serum level of 0.3–4.0 ng/ml (24). We measured three pooled samples (two dams in each pool) and detected unconjugated (free) BPA levels of 4.6, 3.9, and 2.0 ng/ml. These BPA levels fall within the range of normal exposure. By comparison, serum BPA concentrations in pregnant dams on the control phytoestrogen-free diet were below the level of quantification, at 0.04 ng/ml, and only quantifiable by extrapolation from the standard curve.

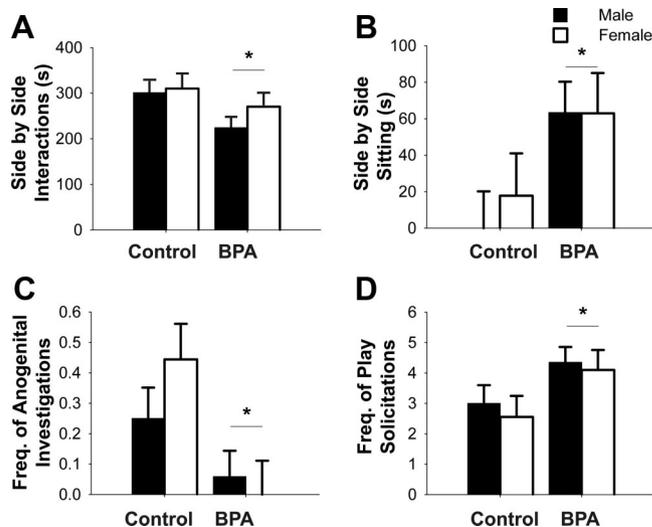


FIG. 1. Mean \pm SEM behaviors displayed during a 10-min reciprocal social interaction task. Juvenile male and female mice were exposed to control diet (phytoestrogen-free chow, $n = 12$ males, black bars; $n = 9$ females, white bars) or BPA-supplemented diet ($n = 17$ males, black bars; $n = 11$ females, white bars) during gestation. A, Duration of side-by-side interactions in seconds (s); B, duration of side-by-side sitting in seconds; C, frequency (Freq.) of anogenital investigations; D, numbers of play-soliciting events. *, Significantly different from control diet, $P < 0.05$. Bars indicate significance is a main effect.

Gestational exposure to BPA affects juvenile social interactions in juvenile mice

Juvenile mice were assessed for social and nonsocial behaviors in an open-ended dyadic social interaction task. Gestational exposure to BPA elicited a number of alterations in behavior in juvenile mice (Fig. 1). BPA-exposed mice spent more time sitting next to each other than did control diet mice [diet effect, $F_{(1,44)} = 6.85$; $P < 0.02$]. Interestingly, while sitting side by side, control mice interacted with each other more than BPA-exposed mice [diet effect, $F_{(1,44)} = 4.18$, $P < 0.05$]. Investigative anogenital sniffing [diet effect, $F_{(1,44)} = 9.30$, $P < 0.004$] was reduced in mice exposed to BPA as compared with controls. Play-soliciting behavior (the sum of numbers of crawls, pushes, and approaches) was significantly higher in BPA vs. control diet mice [diet effect, $F_{(1,44)} = 5.53$, $P < 0.03$]. The composite scores of social, nonsocial, and investigative behaviors were not significantly altered by BPA. It is important to note that none of these behaviors proved to be sexually dimorphic, nor did we find any interactions between sex and diet.

Gestational BPA exposure decreases social preference for an adult male

In a three-chambered social preference task, juvenile males exposed *in utero* to BPA spent less time with an adult male conspecific [sex by diet interaction, $F_{(1,47)} = 8.3$, $P < 0.006$] and more time in the empty chamber [sex by diet interactions, $F_{(1,47)} = 8.74$, $P < 0.005$, Fig. 2] than did

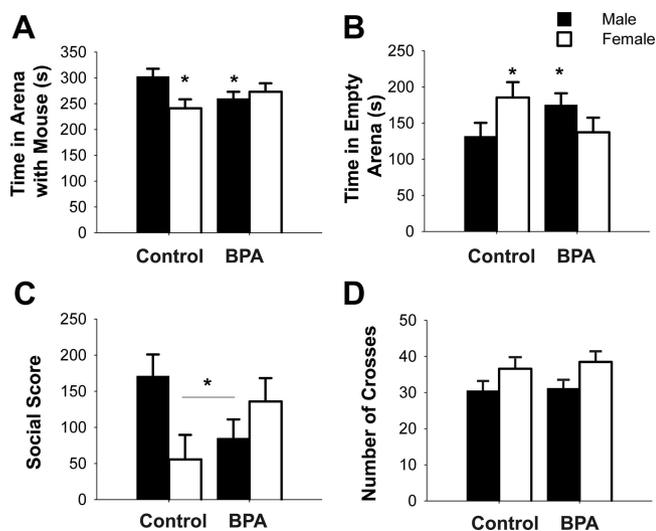


FIG. 2. Mean \pm SEM behaviors displayed during a 10-min social preference task in juvenile mice exposed to control diet ($n = 12$ males, black bars; $n = 9$ females, white bars) or BPA-supplemented diet only during gestation ($n = 17$ males, black bars; $n = 11$ females, white bars). A, Time spent on the side with another mouse in seconds (s); B, time spent in the empty chamber; C, social difference score; D, number of crosses between the chambers. *, Significantly different from control males.

control males. The social score, calculated as the time spent in the chamber with the mouse minus the time spent in the empty chamber, also revealed a significant interaction between diet and sex [$F_{(1,47)} = 9.72, P < 0.004$]. In this case, BPA-exposed males and control females were less social than control males or females exposed to BPA in their dams' diet. Thus, the sex difference noted in mice exposed to control diet is switched by BPA exposure. BPA females spent less time in the empty chamber, thus producing social scores similar to control males. The amount of activity, assayed by number of crosses between chambers, was not influenced by sex or diet, nor was the amount of time spent sniffing either the empty or mouse-containing cylinders.

BPA effects on social interaction in juvenile F_2 and F_4

In general, BPA exposure increased social behavior and decreased nonsocial behavior in F_2 mice, and these patterns persisted into the fourth generation (Fig. 3). This is in contrast to the first-generation tests in which BPA exposure decreased social preference and interactions and increased play-soliciting behavior. Juvenile mice from the F_2 BPA lineage spent more time engaging in side-by-side interactions [diet effect, $F_{(1,76)} = 9.5, P < 0.003$] than did mice in the control line. A diet effect was observed for exploring the cage [$F_{(1,76)} = 4.42, P < 0.04$]; no interactions or sex differences were found for either behavior. An interaction between diet and sex was found for self-

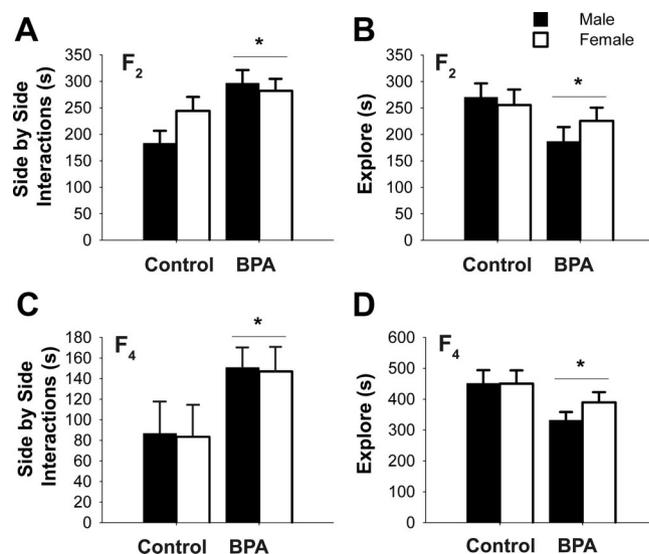


FIG. 3. Mean \pm SEM behaviors displayed by F_2 and F_4 mice during a 10-min reciprocal social interaction task. Juvenile male and female mice were descendants of mice exposed to control diet ($F_2, n = 21$ males and $n = 17$ females; $F_4, n = 7$ males and $n = 7$ females) or BPA-supplemented chow ($F_2, n = 19$ males and $n = 23$ females; $F_4, n = 19$ males and $n = 12$ females). Black bars, male mice; white bars, female mice. A, Duration in seconds (s) of side-by-side interaction in F_2 mice; B, total amount of time (seconds) spent exploring the cage in F_2 mice; C, duration of side-by-side interaction in F_4 mice; D, total amount of time spent exploring the cage in F_4 mice. *, Significantly different from control diet lineage, $P < 0.05$.

grooming [$F_{(1,76)} = 4.6, P < 0.04$], which was elevated in females in the BPA lineage and equaled that of males in the control lineage (Fisher least significant difference *post hoc*, $P < 0.05$). Other social behaviors, which were influenced by diet in the F_1 mice, for example, side by side sitting, anogenital investigation, and play solicitations, were not significantly affected by sex or diet in the F_2 mice.

Many of the diet-related differences in juvenile social behavior present in the F_2 mice were also found in the fourth generation. Because the F_4 mice were never directly exposed to dietary BPA, we conclude that these effects were transmitted through the germline. Side-by-side interactions were almost 2-fold higher in the BPA lineage mice over the controls [diet effect, $F_{(1,40)} = P < 0.02$]. When all social behaviors were added together to make a composite score, we noted a trend for more social activities in the BPA lineage than in the controls [diet effect, $F_{(1,40)} = 4.09, P < 0.06$]. A main effect of diet was also noted for time spent exploring, a nonsocial behavior [$F_{(1,40)} = 5.54, P < 0.02$; Fig. 3]. The composite score for nonsocial behaviors similarly tended to be lower in the BPA lineage compared with controls [diet effect, $F_{(1,40)} = 3.85, P < 0.06$]. As noted in the F_2 generation, play-soliciting and investigative behaviors were unaffected in the F_4 cohorts.

Interestingly, no diet or sex effects on any social preference measures were found in the F_2 generation (Supple-

TABLE 1. Elevated plus maze (mean \pm SEM) in juvenile F₁, F₂, and F₄ mice

Group	Time in open arm (sec)	Time in distal open arm (sec)	Time in closed arm (sec)	Time in center (sec)	No. of crosses	
F ₁	Control male, n = 10	76.1 \pm 11	29.8 \pm 6	375.3 \pm 20	148.6 \pm 10	25.4 \pm 2
	Control female, n = 10	62.6 \pm 14	25.3 \pm 8	414.4 \pm 19	123 \pm 10	25 \pm 3
	BPA male, n = 18	77.6 \pm 7	36.2 \pm 4	388.5 \pm 11	133.9 \pm 6	25.1 \pm 2
	BPA female, n = 11	64.2 \pm 12	28.8 \pm 5	412.0 \pm 22	123.8 \pm 12	19.8 \pm 3
F ₂	Control male, n = 24	78.1 \pm 5	34.7 \pm 3	406.1 \pm 10	204.4 \pm 24	22.2 \pm 1
	Control female, n = 20	86.4 \pm 9	40.4 \pm 5	413.6 \pm 11	249.2 \pm 30	23.3 \pm 2
	BPA male, n = 13	100.8 \pm 7	48.5 \pm 4	377.8 \pm 10	258.8 \pm 20	23.8 \pm 1
	BPA female, n = 19	97.9 \pm 7	45.7 \pm 4	386.5 \pm 8	235.2 \pm 20	27.4 \pm 2
F ₄	Control male, n = 13	109.5 \pm 11	47.2 \pm 8	351.1 \pm 21	139.5 \pm 13	25.8 \pm 2
	Control female, n = 20	101.4 \pm 10	46.2 \pm 6	368.7 \pm 18	130.0 \pm 10	27.3 \pm 2
	BPA male, n = 24	95.6 \pm 9	41.3 \pm 4	377.8 \pm 12	126.5 \pm 7	26.3 \pm 1
	BPA female, n = 18	88.6 \pm 11	43.2 \pm 7	414.2 \pm 13	97.2 \pm 9	23.9 \pm 2

mental Fig. 1, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Because of this, we did not test the F₄ generation in this task. Importantly, gestational BPA did not alter anxiety-like behavior at any generation in the elevated plus maze. The time spent in the open arms and closed arms and the number of crosses between arms were similar in all groups (Table 1).

First-generation effects of BPA on genome-wide gene expression

After correction for multiple comparisons (q-values), we found only one significant transcript change based on diet, caspase 9 (*Casp9*), an initiator of the intrinsic apoptotic pathway (Supplemental Table 1) from our genome-wide microarray analysis. Interestingly, BPA increases this gene in testes (55) as it appeared to here in the brain. The lack of robust gene expression differences between BPA-exposed and control animals may be due to the fact that whole brain was used for our analysis where gene responses in different brain regions could cancel each other out. Gene changes restricted to small discrete brain nuclei would less likely be detected in these analyses. Uncorrected *P* values, however, indicated that BPA decreased estrogen receptor α (*Esr1*, *P* = 0.0009), vasopressin (*Avp*, *P* = 0.003), and vasopressin receptor (*Avpr1a*, *P* = 0.003). Given the importance of these molecules in social behavior, the fact that BPA binds to and activates estrogen receptors (56–58), and the possibility of false negatives with correction for multiple comparisons, we further examined expression of these molecules and other candidate genes using qPCR. Four transcripts were significantly altered by sex and increased in females after correction for multiple comparisons (Supplemental Table 2). Three of these transcripts lie within the X-inactive specific tran-

script, *Xist*, and one lies within another gene, lysine (K)-specific demethylase 5d, *Kdm5d*, the Y homolog of an X-linked gene known to escape X-inactivation (59). Expression of both *Xist* and *Kdm5d* are known to differ between females and males. No transcripts were significantly altered for the interaction between diet and sex after corrections for multiple comparisons (Supplemental Table 3).

Effect of gestational BPA on estrogen receptors and related genes by qPCR

Gestational exposure to BPA had direct effects on several estrogen receptors in the developing brain. We confirmed expression of *Esr1* and estrogen receptor β (*Esr2*), the G protein-coupled membrane-bound estrogen receptor (*Gper*, commonly known as *GPR30*) and estrogen-related receptor γ (*Esrrg*), on E18.5 in whole brain using qPCR (Fig. 4). BPA exposure during gestation decreased *Esr1* expression in embryonic brain [diet effect, $F_{(1,8)} = 12.23$, *P* < 0.009]. There were no sex differences, nor was there an interaction between sex and diet. Conversely, BPA-exposed embryos had higher levels of *Gper* and *Esrrg* [diet effect, $F_{(1,8)} = 11.42$, *P* < 0.01; and $F_{(1,8)} = 8.16$, *P* < 0.03] mRNA than controls. A sex difference in *Gper* was also detected; males have higher concentrations than females [sex effect, $F_{(1,8)} = 13.96$, *P* < 0.006]. A trend for an interaction between diet and sex was found for *Esrrg* [$F_{(1,8)} = 5.22$, *P* = 0.054] and *Gper* [$F_{(1,8)} = 4.80$, *P* = 0.06]. For both genes, males exposed to BPA had the highest gene expression. *Esr2* was not significantly affected by diet or by sex. Increased *Casp9* expression in BPA-exposed mice found in the microarray were not confirmed by qPCR, indicating that this gene is likely a false positive in the microarray dataset. This finding highlights the impor-

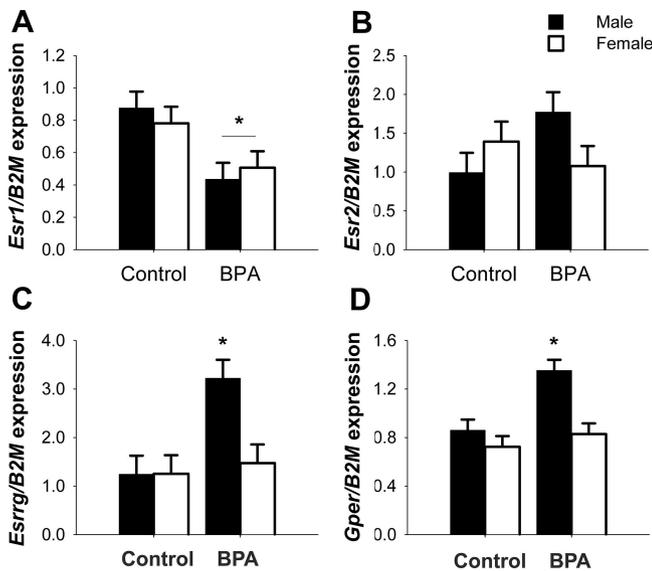


FIG. 4. Mean \pm SEM mRNA levels in the embryonic (E18.5) brain quantified by qPCR. All values are relative to control males and normalized to β 2-microglobulin ($n = 3$ per group). *Black bars*, male mice; *white bars*, female mice. A, Estrogen receptor α (*Esr1*); B, estrogen receptor β (*Esr2*); C, estrogen-related receptor γ (*Esrg*); D, G protein-coupled estrogen receptor (*Gper*). *, Significantly different from control diet, $P < 0.05$. Bars indicate significance is a main effect.

tance of confirmation of microarray findings by another independent method such as qPCR.

Avp expression was 4-fold less in F_1 embryonic brains exposed to BPA [diet effect, $F_{(1,8)} = 14.45$, $P < 0.006$]; no sex effects or interactions were present (Fig. 5). None of the other genes we examined were significantly affected by

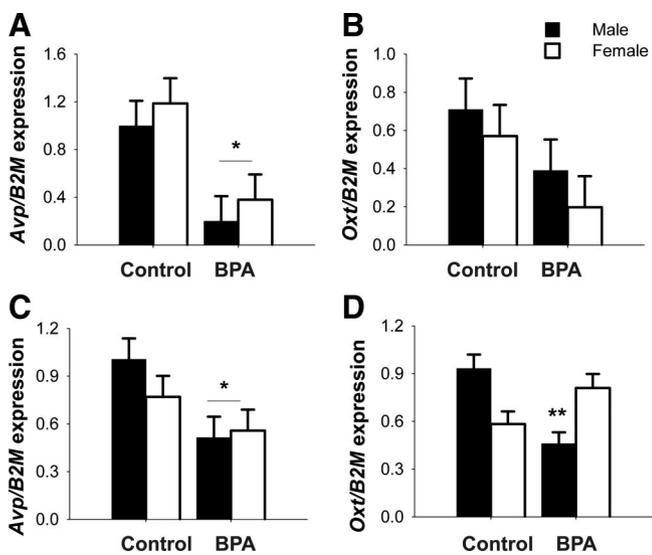


FIG. 5. Mean \pm SEM mRNA for genes in the embryonic (E18.5) whole brains of mice in the F_1 and F_4 generations quantified by qPCR. Values are relative to control males and normalized to β 2-microglobulin ($n = 5$ – 6 per group). *Black bars*, male mice; *white bars*, female mice. A, Arginine-vasopressin (*Avp*) in F_1 ; B, oxytocin (*Oxt*) in F_1 ; C, arginine-vasopressin (*Avp*) in F_4 ; D, oxytocin (*Oxt*) in F_4 . *, Significantly different from control diet, $P < 0.05$; **, significantly different from males in the control diet lineage, $P < 0.05$.

diet or sex. However, we noted a trend for lower *Oxt* mRNA in BPA vs. control brains [diet effect, $F_{(1,8)} = 4.50$, $P = 0.066$], and in contrast its receptor, *Oxtr*, tended to be increased by BPA [diet effect, $F_{(1,8)} = 4.44$, $P = 0.06$; Table 2]. We previously found that expression of the glutamate transporter *Slc1a1* was elevated in brains of female embryos exposed to BPA (18). At the present higher dose, BPA exposure decreased *Slc1a1* expression as compared with controls [$F_{(1,8)} = 8.09$, $P = 0.02$; Table 2]. Although caspase 9, *Casp9*, was increased in whole brain of males and females exposed to BPA in the microarray dataset, PCR was not able to detect any diet effect (Table 2). Lastly, we measured *Dnmt1*, *Dnmt3a*, and *Dnmt3b*, but transcript expression of these genes was not significantly affected by BPA diet or sex ($P > 0.05$; Table 2).

Transgenerational effects of BPA on *Avp* and *Oxt* gene expression

Genes significantly altered by BPA exposure in the first generation were measured by qPCR within the fourth generation to determine potential transgenerational effects of BPA exposure. None of the estrogen receptor genes, caspase 9, or the glutamate transporter were significantly different by sex or lineage in the F_4 mice (Table 2). However, *Avp* expression levels were significantly decreased in the BPA lineage compared with controls [diet effect, $F_{(1,8)} = 7.1$, $P < 0.03$], similar to results seen in the F_1 mice (Fig. 5). As in the first generation, no sex differences were present. In addition, *Oxt* mRNA levels revealed an interaction between BPA and sex [$F_{(1,15)} = 17.92$, $P < 0.001$]. In the F_1 brains, this interaction was present as a trend. In the F_4 brains, males in the BPA lineage had decreased *Oxt* expression compared with control males and females of the BPA line (Fisher least significant difference *post hoc*, $P < 0.05$). Together, these data suggest that BPA-induced expression changes in social neuropeptides, *Avp* and *Oxt*, are transgenerationally inherited, whereas alterations in estrogen receptor genes are a response to direct exposure to BPA.

Discussion

Here we demonstrate transgenerational effects of BPA on behavior and on mRNA levels of two neuropeptides, vasopressin and oxytocin, both of which are influential in expression of many social behaviors (3, 4). In the BPA-exposed first generation, we observed decreased social interactions in mice of both sexes; side-by-side interactions and preference for an adult male were decreased. These changes in social behavior are likely not caused by differences in anxiety because BPA exposure did not affect be-

TABLE 2. Normalized gene expression levels in F₁ and F₄ E18.5 brains relative to the control male group

Gene	Control male	Control female	BPA male	BPA female
F ₁				
<i>Avpr1a</i>	0.98 ± 0.02	0.94 ± 0.08	1.15 ± 0.21	0.88 ± 0.03
<i>Oxtr</i>	0.94 ± 0.11	1.38 ± 0.35	1.58 ± 0.09	1.62 ± 0.16
<i>Sl1a1</i>	0.99 ± 0.03	1.00 ± 0.05	0.80 ± 0.03	0.95 ± 0.05
<i>Casp9</i>	1.02 ± 0.01	0.96 ± 0.08	0.99 ± 0.04	0.96 ± 0.05
<i>Dnmt1</i>	1.08 ± 0.13	0.93 ± 0.08	1.25 ± 0.08	1.22 ± 0.19
<i>Dnmt3a</i>	1.41 ± 0.22	1.30 ± 0.13	1.18 ± 0.06	1.38 ± 0.23
<i>Dnmt3b</i>	0.88 ± 0.07	0.90 ± 0.05	1.06 ± 0.00	0.97 ± 0.19
F ₄				
<i>Esr2</i>	1.05 ± 0.12	0.78 ± 0.08	1.06 ± 0.14	1.12 ± 0.14
<i>Esr1g</i>	0.96 ± 0.30	0.75 ± 0.08	0.95 ± 0.20	0.89 ± 0.13
<i>Avpr1a</i>	0.79 ± 0.08	0.70 ± 0.04	0.74 ± 0.05	0.73 ± 0.06
<i>Oxtr</i>	1.12 ± 0.10	0.84 ± 0.14	1.17 ± 0.14	1.02 ± 0.09
<i>Slc1a1</i>	0.92 ± 0.07	1.03 ± 0.08	1.09 ± 0.15	1.23 ± 0.07
<i>Casp9</i>	1.31 ± 0.22	1.28 ± 0.14	1.13 ± 0.10	1.33 ± 0.12

Results are shown as mean ± SEM of transcripts normalized to *Ppib* (n = 3–5 per group).

havior in the elevated plus maze. We suspect that differential expression of genes such as *Esr1* (and others we did not measure) in the brains of mice exposed to BPA *in utero* (F₁) affected some of the behaviors we observed. Increases, decreases, or no change in *Esr1* brain expression levels have been reported after pre- or perinatal exposure to BPA (26, 28, 60–62).

Estrogens, acting via nuclear receptors (α or β), have strong effects on many social behaviors in mice of both sexes (63–67). As adults, estrogen receptor α knockout mice have impairments in social investigation of other adults (68), social transmission of food preferences (69), aggression (70), and sexual behavior (71, 72). Here, we examined behavior in juvenile mice at a time when estrogen and androgen levels are very low in plasma (73). Thus, we assume that the differences in behavior found in the first generation might normally be caused by estrogen exposure to males only *in utero*. Fetal exposure to BPA during brain development could disrupt the normal sexual dimorphism in hormone levels *in utero*. Abundant data show that neonatal BPA disrupts normal brain sexual differentiation (25–27). Our data are comparable to those collected in rats wherein juvenile play behavior in males was reduced by perinatal BPA (33, 34). In a previous study, employing a 4-fold lower dose of BPA but the identical treatment methods and times, we found an increase in side-by-side interactive behaviors in female mice exposed to BPA but no changes in any estrogen receptor genes (18). Interestingly, a 10-fold higher dose of BPA than the one used here had a similar effect on social preferences, decreasing behavior in the males exposed to BPA (17). These data highlight the importance of BPA dose and non-monotonic actions of BPA on behavioral and other endpoints (74). More evidence of dose-related effects comes from our previous work in which the transcript levels of

three genes were altered in the brains of females exposed to a lower dose during gestation (18). Two DNA methyltransferases, *Dnmt1* and *Dnmt3a*, as well as the glutamate transporter gene *Slc1a1* were differentially expressed at a 4-fold lower dose of BPA but were not altered in the current study.

Estrogen receptors affect social behaviors via actions as transcription factors. Estrogen receptor α regulates transcription of vasopressin (75) but only indirectly modulates oxytocin (65). The colocalization of *Gper* to oxytocin- and vasopressin-expressing neurons within hypothalamic nuclei suggests that estrogen signaling through this G protein-coupled receptor may also play a role in gene regulation (76, 77). We speculate that the combination of decreased *Esr1* gene expression and increased *Gper* gene expression in the brains of F₁ embryos may have directly affected expression of *Avp*, *Oxt*, and other genes involved in social behavior (65), which in turn likely influenced social behaviors in the F₁ juveniles. The F₁ generation was directly exposed to BPA *in utero*, and differences in expression of the estrogen receptors were detected only in that generation. Because the F₄ generation had no direct exposure to BPA, it is likely that differences in behavior between mice in the two different diet lineages reflects the actions of estrogen receptors that were modified by diet in the first generation but not in the F₄ mice. Mice in the F₂ generation, who experienced exposure to BPA as gametes, showed increased social interactions along with decreased nonsocial behaviors such as exploring the cage and self-grooming. Importantly, the differences and the direction of these effects in F₂ mice persisted into the fourth generation.

We hypothesize that the persistent changes in *Avp* and *Oxt* detected in brains from the F₄ generation occur via a heritable epigenetic process, such as DNA methylation.

Others have shown that DNA methylation within regulatory regions of *Avp* can be modified by estrogens (78) or early life stress (79). Alternatively, other types of epigenetic modifications such as alterations of histones, chromatin modifications, or noncoding RNA may explain the heritable actions of BPA on gene expression and behavior. Because both oxytocin and vasopressin have very discreet neural distributions, we are now assessing which subpopulations of these neurons are influenced by BPA as well as the mechanism of action.

Previous studies have demonstrated that BPA can have trans- or multigenerational effects on other genes in other tissues (20, 23), and exposure to a range of man-made steroid-like substances, including vinclozolin (12), diethylstilbestrol (11, 13), methoxychlor (12), and chromium (10), can result in defects that persist through generations. To our knowledge, there is only one other report of transgenerational actions of an EDC on behavior. Rats exposed to vinclozolin from E8–14 have disrupted spermatogenesis as well as changes in adult mate preference and anxiety behavior (14, 15). Recent work on BPA-induced alterations in the methylome suggest multiple genes in tissues derived from different germ layers are differentially methylated, suggesting that these changes occurred early on, perhaps at the level of the germ cell (19–22). These types of epigenetic changes can produce transgenerational effects.

In sum, we have demonstrated for the first time to our knowledge that a common and widespread EDC has transgenerational actions on social behavior and neural expression of at least the genes for vasopressin and oxytocin. We exposed mice to BPA throughout gestation to include the period of epigenetic resetting of DNA methylation within the germline. During the time of exposure, BPA alters expression of several estrogen receptor, oxytocin, and vasopressin genes in the brains of embryonic mice. Some of these changes are likely inherited via epigenetic mechanisms through the germline, allowing effects to last until the fourth generation. Because exposure to BPA changes social interactions at a dose within the reported human levels, it is possible that this compound has transgenerational actions on human behavior.

Acknowledgments

We thank Aileen Ryalls for her technical assistance with animal husbandry and Drs. Charles Farber and Achilleas Pitsillides for evaluation of the expression analysis dataset.

Address all correspondence and requests for reprints to: Dr. Emilie Rissman, University of Virginia, P.O. Box 800733, Charlottesville, Virginia 22908. E-mail: rissman@virginia.edu.

This work was supported by NIMH R01 MH086711 (to E.F.R.), Autism Speaks Grant 4802 (to E.F.R.), and Cardio Vascular Research Center startup funds (to J.J.C.). J.T.W. is supported by NIH Grant F32 ES019404.

Disclosure Summary: The authors have no competing financial interests to declare.

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