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Humans are routinely exposed to bisphenol A (BPA), an estrogenic chemical present in food and beverage containers, dental composites, and many products in the home and workplace. BPA binds both classical nuclear estrogen receptors and facilitates membrane-initiated estrogenic effects. Here we explore the ability of environmentally relevant exposure to BPA to affect anatomical and functional measures of brain development and sexual differentiation. Anatomical evidence of alterations in brain sexual differentiation were examined in male and female offspring born to mouse dams exposed to 0, 25, or 250 ng BPA/kg body weight per day from the evening of d 8 of gestation through d 16 of lactation. These studies examined the sexually dimorphic population of tyrosine hydroxylase (TH) neurons in the rostral periventricular preoptic area, an important brain region for estrous cyclicity and estrogen-positive feedback. The significant sex differences in TH neuron number observed in control offspring were diminished or obliterated in offspring exposed to BPA primarily because of a decline in TH neuron number in BPA-exposed females. As a functional endpoint of BPA action on brain sexual differentiation, we examined the effects of perinatal BPA exposure on sexually dimorphic behaviors in the open field. Data from these studies revealed significant sex differences in the vehicle-exposed offspring that were not observed in the BPA-exposed offspring. These data indicate that BPA may be capable of altering important events during critical periods of brain development. (Endocrinology 147: 3681–3691, 2006)
and spatial control of estrogen action during critical periods of brain development (37). These include the transient expression of both estrogen receptors and aromatizing enzymes that correspond to transient increases in testosterone production in the developing male. In rodents and other animal models, numerous studies have documented that testosterone secreted by the testes in the developing male is converted to estradiol in situ by aromatase, which is present in specific brain regions during critical periods of perinatal development. This conversion of testosterone to estradiol plays an important role in the sexual differentiation of the brain (for review see Refs. 37 and 38). Unlike the testes, the ovaries of the developing female rodent are quiescent peri-natally (39). Recent data have verified the importance of α-fetoprotein (Afp) in protecting the brain of the female fetus from defeminization or masculinization by binding circulating estradiol from the mother or neighboring male littermates (40). Afp, which is abundant during fetal and early neonatal life (41, 42), binds estradiol in rodents with high affinity. Nonsteroidal estrogens, such as BPA, that exhibit a lower affinity for plasma estrogen-binding proteins (43) may be able to evade this protective mechanism.

In the present studies, we explore the ability of chronic low-level perinatal exposure to BPA to affect anatomical and functional measures of brain development and sexual differentiation. First, we searched for anatomical evidence of alterations in brain sexual differentiation. For these studies, we concentrated on a prominent sexually dimorphic region of the brain in the rostral periventricular preoptic area. The anteroventral periventricular preoptetic area (AVPV) is important for cyclic gonadotropin release and for the LH surge required for ovulation (44, 45). Neurons in this region contain sexually dimorphic patterns of steroid receptor distribution (46) and peptide expression in rats (47–49). One robust sex difference that has been observed in the AVPV of both rats (46) and peptide expression in rats (47–49). One robust sex difference that has been observed in the AVPV of both rats and mice is the sexually dimorphic population of tyrosine hydroxylase (TH) neurons (50–52). TH is the rate-limiting enzyme for dopamine synthesis. The number of TH-positive neurons in the AVPV is significantly higher in female rats and mice relative to males, and the sexual dimorphism of this population of neurons appears to be dependent on perinatal levels of gonadal steroids (50, 51). Studies of estrogen receptor knockout (ERKO) mice indicate that the significant decline in TH neuron number in the male AVPV is dependent upon the presence of ERα (52). To determine whether perinatal BPA exposure can alter a robust anatomical marker of brain sexual differentiation in mice, we compared TH neuron number in tissue sections through the rostral periventricular preoptic area in male and female littersmates born to mothers exposed to BPA or to vehicle only. Herein we report effects of perinatal BPA exposure on the expected sex differences in TH neuron number.

As a second and functional endpoint of BPA action on brain sexual differentiation, we examined potential effects of developmental exposure to BPA on sexually dimorphic behaviors in the open field. Strain-dependent sex differences have been reported in open-field behaviors in rats and mice (53, 54), and data from the majority of studies reveal higher levels of activity in females relative to males (54–56). Although circulating hormones in adulthood can affect these behaviors, differential exposure to gonadal steroids during the perinatal period plays an important role in the development of sexually dimorphic behaviors in the open field (55). More specifically, data from studies of male ERKO mice suggest that masculinization of open-field behavior requires estrogen action during development (57). The results of these behavioral studies further suggest that early exposure to BPA may alter important events during critical periods of brain development in mice and indicate the need for careful study of the potential effects of developmental exposure to this compound in humans.

Materials and Methods

Animals

Animals for these studies were maintained in temperature- and light-controlled (14 h light, 10 h dark, lights on at 0400 h) conditions at the Tufts-New England Medical Center Animal Facility. The cages and bedding used for housing tested negligible for estrogenicity using the E-SCREEN assay (58). The food (rodent diet 2018; Harlan Teklad, St. Louis, MO) was extracted and assayed as previously described (58), and it also tested negligible for estrogenicity (20 fmol estradiol equivalents/g). Water was supplied by glass bottles only. All experimental procedures were approved by the Tufts University-New England Medical Center Animal Research Committee in accordance with the Guide for Care and Use of Laboratory Animals.

To generate offspring for these studies, female and male CD-1 mice were purchased (Charles River Laboratories, Wilmington, MA) at 8–10 wk of age. Female breeders were housed between cages of breeder males for a minimum of 1 wk after arrival. Two days before introduction of a male into the cage, females were exposed to male bedding to stimulate estrous cyclicity and ovulation. Males were placed in the female cages, and the morning on which a vaginal plug was observed was designated d 1 of pregnancy. On the evening of d 8 of pregnancy, dams were weighed and implanted sc with Alzet osmotic pumps (Alza Corp., Palo Alto, CA) that were designed to deliver either 50% dimethylsulfoxide (vehicle control) or BPA dissolved in 50% dimethylsulfoxide at the rate of 25 ng or 250 ng BPA/kg body weight (BW) per day (Sigma Chemical Co., St. Louis, MO) throughout the remainder of the pregnancy and through d 16 of lactation. Dams were allowed to deliver naturally, and the litters were culled to eight pups per mother (four males and four females) d 1 after birth. Litters were weaned on postnatal d 22–24. Only litters with normal distributions of males and females were included in these studies. The average number of pups and mean percentage of female pups in each litter examined in these studies are shown in Table 1.

Vehicle-exposed and BPA-exposed offspring for anatomical studies were examined before puberty (22–24 d). For behavioral studies, offspring were initially examined at 6–9 wk of age. However, because of concerns regarding potential effects of differences in circulating hormone levels in postpubertal animals, additional offspring were examined before puberty (27–29 d of age). It should be noted that vaginal smear records from our colony indicate that 6- to 9-wk-old group-housed females do not exhibit regular 4- to 5-d estrous cycles (Rubin, B. S., and A. M. Soto, unpublished data). This observation probably reflects the importance of pheromones to the maintenance of regular estrous cyclicity and ovulation. Males were placed in the female cages, and the morning on which a vaginal plug was observed was designated d 1 of pregnancy. On the evening of d 8 of pregnancy, dams were weighed and implanted sc with Alzet osmotic pumps (Alza Corp., Palo Alto, CA) that were designed to deliver either 50% dimethylsulfoxide (vehicle control) or BPA dissolved in 50% dimethylsulfoxide at the rate of 25 ng or 250 ng BPA/kg body weight (BW) per day (Sigma Chemical Co., St. Louis, MO) throughout the remainder of the pregnancy and through d 16 of lactation. Dams were allowed to deliver naturally, and the litters were culled to eight pups per mother (four males and four females) d 1 after birth. Litters were weaned on postnatal d 22–24. Only litters with normal distributions of males and females were included in these studies. The average number of pups and mean percentage of female pups in each litter examined in these studies are shown in Table 1.

Table 1. Composition of the litters used for the studies described

<table>
<thead>
<tr>
<th>BPA treatment</th>
<th>Mean no. of pups per litter</th>
<th>Proportion of female pups per litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.17 ± 0.58</td>
<td>0.591 ± 0.026</td>
</tr>
<tr>
<td>25 ng</td>
<td>11.30 ± 0.423</td>
<td>0.524 ± 0.052</td>
</tr>
<tr>
<td>250 ng</td>
<td>11.27 ± 0.506</td>
<td>0.524 ± 0.034</td>
</tr>
</tbody>
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No significant differences were noted between any experimental group relative to the controls. All litters were culled to eight pups (four males and four females) d 1 after birth.
Estrous cyclicity in mice, which is enhanced by exposure to males or male urine and can be suppressed by exposure to other females (59–61).

**TH neuron number**

Tissue preparation. Two male and two female littermates from each litter were killed at 22–24 d of age. Prepubertal animals were chosen for examination because of evidence of a potential influence of circulating steroid levels on TH expression in adult animals (50). Animals were injected with an overdose of pentobarbital and perfused intraventricularly with heparinized saline followed by a solution containing 4% paraformaldehyde and 3.0% acrolein. Brains were removed from the calvarium, placed into phosphate buffer, and stored at 4 C. Brains from a matched set of male and female littermates from seven to eight different litters were analyzed for each of the three exposure levels (ranging from 0–250 ng BPA/kg BW/d) as detailed above.

**Immunocytochemistry protocols**

Brains were blocked, and 40-μm sections were cut in the coronal plane on a Vibratome (Technical Products International, St. Louis, MO). Sections were collected beginning rostrally at the diagonal band of Broca and continuing caudally through the arcuate nucleus. Consecutive brain sections were collected into two numbered tissue boats with each boat receiving every other section through the region of interest, thus limiting the number of tissue sections in each boat to facilitate antibody incubations. Both tissue boats for each animal were treated identically so that every section through the areas of interest was available for analysis. After pretreatment to remove residual aldehydes, tissues were rinsed, and free-floating sections were incubated for 48 h at 4 C in anti-TH monoclonal antibody (MAB318; Chemicon, Temecula, CA) diluted at 1:5000. Secondary antibody was biotin-SP-conjugated donkey antiamouse (no. 715-066-151; Jackson ImmunoResearch, West Grove, PA). Detection was completed with Vectastain ABC reagent (PK-4000; Vector Laboratories, Burlingame, CA) and diaminobenzidine. A total of seven chemistries were performed, and each immunocytochemistry run included tissues from matched male and female littermates from the different treatment groups.

**Data analysis**

Tissue sections through the AVPV were identified using the Mouse Brain Atlas of Paxinos and Franklin (62) as a guide to define the parameters of the region of interest. The region analyzed corresponds to that identified as the AVPV in Figures 26–30 in the atlas and is indicated by the shaded areas in Fig. 1. As depicted, this region extends from interaural 4.42 mm to interaural 3.94 mm, with the consecutive sections examined beginning rostrally at the level of the organum vasculosum of the lamina terminalis and extending caudally to the level just before the crossing of the anterior commissure. TH neurons were identified using a Zeiss Axioscope (×10 and ×40 objectives), and counted by three different observers blind to the sex and treatment groups of the animals.

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Endocrinology, August 2006, 147(8):3681–3691

**FIG. 1.** Sections through the rostral preoptic area. The drawings depicted were adapted from the Mouse Brain Atlas by Paxinos and Franklin (62), and the AVPV, as defined by the atlas, appears shaded. As shown, these drawings span the region extending from interaural 4.42 mm rostrally through interaural 3.94 mm caudally. Actual tissue sections through most (nine of 11) of the rostral-caudal extent of the AVPV from one representative female in the study are also shown below the adapted atlas diagrams. The tissue sections depicted here span an area beginning in the rostral AVPV through the region before the crossing of the anterior commissure. 3V, Third ventricle; aca, anterior commissure; VOLT, organum vasculosum of the lamina terminalis; MPA, medial preoptic area; MnPO, medial preoptic nucleus.
Because the total number of sections through the AVPV was not identical for all animals in the study, TH neuron number was also assessed in seven consecutive sections extending from the caudal AVPV (rostro to the level of the crossing of the anterior commissure) through the mid-region of the AVPV rostrally in each animal. TH neuron number was also assessed in two matched sections through the arcuate nucleus of the hypothalamus.

Behavioral studies

Open-field test at 6–9 wk of age. At 6–9 wk of age, male and female offspring of pregnant dams treated with 0, 25, or 250 ng BPA/kg BW/d were observed in a novel open field. The open-field apparatus was a large plastic tub measuring 16 × 24 inches with a wall height of 11 inches. The floor of the tub was divided into squares to facilitate behavioral measurements. The apparatus was carefully cleaned with ethanol, rinsed with water, and then dried after each behavioral test. Two identical open fields were available for testing and were rotated during the testing period. All behavioral tests were conducted in the same room at the same time of day (1200–1400 h) and were scored by three observers. Before the start of the data collection, 3 d of behavioral tests were conducted with an additional cohort of animals to familiarize the observers with the behaviors to be recorded and to standardize the scoring of behaviors. A total of 94 animals were tested in the open field, including 14–17 males and females from each of the three treatment groups. Only a single male and female from each litter were examined in these studies to eliminate potential litter effects. At the start of each test, the mouse was carefully placed into the center of the open field and his/her movements were recorded over the next 5 min. Measurements included number of rears, time spent in center, time stopped, time grooming, and number of fecal pellets. Initial attempts to score the number of squares entered to assess distance traveled in a given testing period were abandoned because the animals moved too quickly to obtain accurate numbers without digital recordings.

Open-field tests in prepubertal animals. Additional open-field tests were performed in younger prepubertal animals at 27–29 d of age. These animals were examined before vaginal opening and before the establishment of adult gonadal hormone levels. For these studies, control offspring (n = 10 animals per sex) and offspring born to females treated with 250 ng BPA/kg BW/d (n = 12 animals per sex) were examined. Animals were tested in the open field as described above with one change to the protocol. Because the prepubertal animals were very active, we chose to detain each subject in the center of the open field covered by the transfer beaker for a period of 5 sec before the start of the test to facilitate accurate scoring of behaviors. This method has been used by other laboratories. Unfortunately, this procedural change may have influenced the time-in-center measurement such that this parameter was not comparable in the two age groups examined.

Statistics

The data were analyzed using both parametric and nonparametric statistics. Both showed similar significant differences. The parametric analyses are presented, and the data are graphed as mean ± SEM. The anatomical data and the behavioral data from the 6- to 9-wk animals were analyzed by two-way ANOVA with sex and BPA as the two parameters. Planned comparisons of male and female offspring from each treatment group were evaluated by t tests, and planned same-sex comparisons across treatment groups were assessed by ANOVA followed by Bonferroni post hoc tests. Because the behavioral data for the prepubertal animals contained only a single BPA dose, planned comparisons of male and female offspring in the control and BPA-exposed group and same-sex comparisons across the two treatment groups were analyzed by t tests.

Results

TH neuron number in control and BPA-exposed offspring

Mean number of tissue sections through the AVPV. Comparisons of the total number of tissue sections through the AVPV (based on the delineation of this region in the Paxinos and Franklin atlas and independent of the presence or absence of TH neurons) by two-way ANOVA revealed overall significance (P = 0.039) and a significant interaction between variables (sex × BPA, P = 0.014). The number of tissue sections differed significantly in male and female offspring born to vehicle-treated mothers (P = 0.003, t test; see Fig. 2). Tissue section number did not differ significantly in male and female offspring from either of the BPA-exposed groups. These data are consistent with an increase in the rostral-caudal extent of the AVPV in control females relative to control males and a loss of this sex difference in offspring of BPA-treated dams. Comparison of tissue section number by sex across treatment groups revealed a difference in females (P = 0.020, ANOVA), and post hoc analysis revealed significant differences between control females and those exposed to 250 ng BPA (P = 0.023, Bonferroni).

TH-positive cell number. Analysis (two-way ANOVA) of total TH cell number revealed an overall significance (P < 0.001) and differences by sex (P < 0.001), BPA (P = 0.020), and BPA × sex (P = 0.014). Subsequent analyses (t test) revealed that the total number of TH-positive neurons counted in sections through the AVPV differ in male and female offspring born to vehicle-treated mothers (P = 0.001; control male TH neuron number = 46% of control females; see Figs. 3 and 4) and to mothers exposed to the lowest dose of BPA, 25 ng/kg BW/d (P = 0.024; male TH neuron number = 65% of females). No significant sex differences were observed in offspring exposed to 250 ng BPA/kg BW/d (male TH neuron number = 80% of females). Moreover, comparison of females across treatment groups was significant (P = 0.005, ANOVA), and post hoc analysis revealed that females exposed to the higher dose of BPA exhibited a significant decrease in the total number of TH-positive cells relative to control females (P = 0.004, Bonferroni). Females exposed to the low dose of BPA revealed a 24% decrease in TH neuron number and those exposed to the higher dose revealed a 41%
decline in TH neuron number relative to controls. No significant differences in TH cell number were noted across treatment groups in the males.

Measurements of total cell counts included assessment of TH neurons in every section through the AVPV in each animal and therefore did not include equal numbers of sections from every brain. When TH-positive cell counts were restricted to seven consecutive sections through the AVPV of each brain (beginning caudally just before the crossing of the anterior commissure and extending rostrally through the midregion of the AVPV), analysis (two-way ANOVA) revealed overall significance ($P < 0.001$) and significant differences by sex ($P < 0.001$) and by BPA ($P = 0.032$). Additional analyses revealed significant sex differences in control offspring ($P < 0.001$, ANOVA), and $post hoc$ tests revealed that TH neuron number was significantly decreased in females born to mothers treated with the 250-ng dose of BPA relative to controls ($P = 0.010$, Bonferroni). Comparisons of TH-positive neuron number in the more caudal aspect of the AVPV revealed overall significance and differences by sex ($P < 0.001$, two-way ANOVA). Significant sex differences were present in control offspring ($P = 0.001$, $t$ test) and offspring exposed to the lower dose of BPA ($P = 0.005$). The sex difference in animals exposed to 250 ng BPA/kg BW/d approached significance ($P = 0.059$).

TH-positive neurons in the arcuate nucleus. No significant sex differences in the number of TH-positive cells per section were observed in the arcuate nucleus of control or BPA-exposed offspring. There were also no significant differences in cell number in BPA-exposed relative to control offspring (see Fig. 6).

Behavioral tests

Open-field tests in 6- to 9-wk-old animals. Male and female offspring born to mothers exposed to 0, 25, or 250 ng BPA/kg

![Fig. 3. Photomicrographs of sections through the rostral periventricular preoptic area of control and BPA-exposed mice. TH-positive neurons are shown in representative sections through the mid AVPV (A) and through the caudal AVPV (B) of female and male offspring born to control dams and born to dams treated with 250 ng BPA/kg BW/d. III V, Third ventricle; OC, optic chiasm. Bar, 100 μm.](image)

![Fig. 4. Total number of TH-positive neurons in sections though the AVPV. A, Data are shown for offspring born to control dams and offspring born to dams treated with two doses of BPA (mean ± SEM). Significant sex differences are noted in TH neuron number in control offspring (**, $P < 0.001$) as well as offspring born to dams treated with the lowest dose of BPA (*, $P = 0.024$). A significant decline in TH neuron number is noted in 250-ng females relative to controls (**, $P = 0.004$). Black bars, females (n = 7–8 per treatment); gray bars, males (n = 7–8 per treatment). B, The mean female to male ratio of the total TH-positive neuron number was calculated for each pair of littermates examined. As shown, control females have approximately twice the number of TH-positive neurons in the AVPV relative to control males, and the female to male ratio is markedly reduced with exposure to the higher dose of BPA. The x-axis label refers to the level of BPA exposure of the mothers (per kg BW per day).](image)
BW/d were examined in the open field at 6–9 wk of age. Analysis by two-way ANOVA revealed overall significance in three behavioral parameters, and in each case, only the main effect of sex was significant: rears on walls (overall $P = 0.043$; sex, $P = 0.009$), time stopped (overall $P = 0.012$; sex $P < 0.001$), and time in center (overall $P = 0.007$; sex $P < 0.001$; Fig. 7). Additional analysis using planned $t$ tests revealed that male and female offspring born to control dams showed a significant sex difference in all of these parameters ($P_{\text{rears}} = 0.023$; $P_{\text{center}} = 0.005$; $P_{\text{stopped}} = 0.014$). Offspring born to BPA-exposed dams showed no sex difference in these parameters with one exception, time stopped in offspring exposed to the higher dose of BPA ($P = 0.023$). Although the values for BPA-exposed females drifted toward control male values in two parameters (rears and center), no significant differences were revealed by ANOVA in comparisons of females across the three treatment groups.

Behavioral measurements in prepubertal animals. To rule out the possibility that the behavioral differences observed in control and BPA-exposed offspring at 6–9 wk of age resulted from potential differences in circulating hormone levels, prepubertal offspring born to dams exposed to vehicle or 250 ng BPA/kg BW/d were examined in the open field. Two behavioral measurements, rearing behavior and time stopped, revealed significant sex differences in the control animals ($P_{\text{rears}} = 0.010$; $P_{\text{stopped}} = 0.048$, $t$ tests) and not in the BPA-exposed offspring (Fig. 8). BPA-exposed females showed a significant decline in the number of rears relative to control females ($P = 0.028$, $t$ test), which is consistent with a masculinization of this behavior by BPA. The difference in number of rears in control and BPA-exposed males was not statistically significant. In contrast to the behavioral data from the 6– to 9-wk-old animals, sex differences were not observed in the time spent in the center of the open field in control or BPA-exposed prepubertal animals. This measure might have been influenced by the procedural modification at the start of the test. For the older animals, the test period began the moment they were placed in the center of the open field. As mentioned previously, because of the increased activity of the younger animals, they were detained in the center of the open field for a period of 5 sec before the start of the test. This procedural change may have caused some animals to remain in the center of the open field at the start of the test, thus changing the measure from that in the older animals. Alternatively, it is possible that the sex difference in center time emerges later in development. Early assessments of open-field behavior in rats revealed that significant sex differences in activity levels were apparent in behavioral tests conducted at 52 d of age but not in tests conducted 11 d earlier (55). Also, data from behavioral studies in periadolescent mice (33–43 d old) revealed elevated novelty-seeking behaviors in this age group (63) that might have influenced the time-in-center measurement in the younger animals.

Discussion

Perinatal exposure to low levels of BPA alters a marker of brain sexual differentiation

The difference in TH neuron number in the AVPV represents one robust anatomical marker of brain sexual differentiation that has been documented in rats and mice (50–52). Sexual dimorphism in the population of TH neurons in the AVPV appears to result from the influence of sex steroids during the perinatal period of development (50, 64) and is reportedly observed in rat pups by postnatal d 10 (as reviewed in Ref. 38). Perinatal and/or postnatal exposure to testosterone or estradiol is effective in reducing TH neuron number in the AVPV (50, 64). ERKO mice that lack ERα do
not show the sex difference in TH neuron number; however, mice that lack a functioning androgen receptor (Tfm, testicular feminization) do maintain the sexual dimorphism in TH neuron number (52). Therefore, estrogen action through ERα has been postulated to be important for the significant decline in TH neuron number in males relative to females.

As expected, the total number of TH-positive neurons in sections through the rostral-caudal extent of the AVPV was significantly higher in control females relative to males. Offspring born to mothers exposed perinatally to the low dose of BPA (25 ng/kg BW/d) also revealed significant sex differences in total TH neuron number. In contrast, offspring born to mothers exposed to a 10-fold higher dose of BPA (250 ng/kg BW/d) failed to show significant differences in total TH-positive neuron number. When the analysis of TH neuron number was restricted to seven consecutive sections extending from the caudal AVPV through the midregion of the nucleus in all subjects, only the control animals showed a significant sex difference. Additional analysis of the data suggested that TH neurons in the midregion of the AVPV may be particularly vulnerable to perinatal BPA exposure. The loss of sex differences in TH neuron number in BPA-exposed offspring can be attributed primarily to a decrease in TH neuron number in female offspring, which would be consistent with BPA’s actions as an estrogen.

The total number of tissue sections spanning the rostral-caudal extent of the AVPV was greater in female offspring born to vehicle-treated mothers relative to their male littermates. This finding is consistent with previous reports of increased size in the volume of the AVPV in female relative to male rodents (65). No significant sex differences in the rostral-caudal extent of the AVPV were noted in either BPA treatment group. Moreover, females exposed to the higher dose of BPA exhibited a significant decline in section number relative to control females. These data suggest that perinatal BPA exposure may decrease the AVPV volume in the female brain, making it more similar to that observed in the male.

Different mechanisms appear to be responsible for the sexual dimorphism in AVPV volume and the sex difference in AVPV TH neuron number, and BPA exposure may influence both. Studies investigating the involvement of apoptotic genes in sexual differentiation of the nervous system have revealed that overexpression of the cell survival gene Bcl-2 (66) and deletion of the cell death gene BAX (67) decreased cell death in the AVPV and obliterated sex differences in AVPV volume in mice. However, neither manipulation altered the sexual dimorphism in TH neuron number in this nucleus (66, 67). It is possible that other pathways of cell death that do not involve the Bcl-2 family of proteins may be responsible for sex differences in the number of dopamine neurons in the AVPV. Alternatively, developmental exposure to gonadal steroids may influence the differentiation of the dopaminergic cell phenotype in this nucleus. Such a mechanism has been proposed to explain the sexually dimorphic expression of arginine vasopressin in galanin neurons of the principal nucleus of the bed nucleus of the stria terminalis (BST) (68). Like the AVPV, the sex differences in cell number in the BST can be attributed to differential cell death (66, 69). In contrast, the marked sex difference in arginine vasopressin expression in this nucleus has been pro-

**Fig. 6.** TH-positive neurons in the arcuate nucleus. A, Representative sections through the arcuate nucleus of a control female and male showing the distribution of TH neurons. B, Mean values for TH neuron number per section in two sections through the arcuate nucleus of offspring from all treatment groups. As shown, no significant sex differences in TH neuron number were revealed in the arcuate nucleus for any treatment group examined. Black bars, females (n = 4 per treatment); gray bars, males (n = 4 per treatment). The x-axis label refers to the level of BPA exposure of the mothers (per kg BW per day).

**Fig. 7.** Results of behavioral tests conducted in the open field at 6–9 wk of age. Analysis by two-way ANOVA revealed overall significance in three behavioral measurements in the open field. All three revealed significant sex differences in vehicle-exposed offspring. Data are shown here for male and female offspring born to dams exposed to vehicle, 25, or 250 ng BPA/kg BW/d. A, Number of rears at the wall; B, time in center of the open field; C, time stopped. Black bars, females (n = 14–17 per treatment); gray bars, males (n = 14–17 per treatment). The x-axis label refers to the level of BPA exposure of the mothers (per kg BW per day). *, P = 0.023; **, P = 0.014; ***, P = 0.005.
posed to result from the ability of galanin cells to alter their neuronal phenotype in response to perinatal testosterone exposure (68). Whether this sexually dimorphic expression of arginine vasopressin by galanin cells is a result of target-dependent mechanisms that may be explained by marked differences in the projections of the BST in males and females remains to be determined.

**Significance of the AVPV**

The AVPV is essential for the cyclic pattern of gonadotropin release and the generation of the preovulatory LH surge required for ovulation. Lesions of the AVPV abolished spontaneous LH surges and induced persistent vaginal estrus in female rats (44, 45), and antiestrogen implants into this region blocked the steroid-induced LH surge (70). The disruption of normal development and sexual differentiation of this brain region by BPA could contribute to the alterations in estrous cyclicity in adulthood that has been observed in both mice and rats exposed perinatally to this environmental contaminant (13, 20). A subset of neurons in the AVPV project to GnRH neurons in the rostral preoptic area that are thought to be involved in the generation of the LH surge (71–73). Moreover, data from electron microscopy studies have documented synaptic contact between GnRH neurons in the preoptic area and TH-containing axon terminals (74), and there is evidence that the TH fibers that contact GnRH neurons originate from dopamine neurons in the AVPV (75). Although the role of dopamine in the preovulatory LH surge is still not understood, these observations suggest a possible role for TH neurons in the AVPV in the regulation of gonadotropin release.

**BPA exposure and other anatomical markers of brain sexual differentiation**

To date, three studies have reported evidence of alterations in other anatomical markers of brain sexual differentiation after exposure to BPA during pregnancy and lactation. The expected sex difference in the volume of the locus ceruleus was abolished in offspring born to rats dams exposed to 1.5 mg BPA/kg BW/d in their drinking water (30), and the sex difference in the volume of this nucleus was reportedly inverted in offspring born to dams exposed to 30 or 300 μg BPA/kg BW/d (31). Sex differences in the number of CRH neurons in subdivisions of the BST were abolished in offspring born to mothers exposed to 2.5 mg BPA/kg BW/d via drinking water (76). Only one dose of BPA was examined in this study, and therefore it is not possible to assess the sensitivity of this parameter to perinatal BPA exposure. It should be noted that all of the results discussed above were obtained with exposure levels of BPA that were significantly higher than those assessed in the present study.

**Behavioral data reveal a lasting effect of BPA exposure on the developing brain**

Strain-dependent sex differences have been reported in open-field behaviors in rats and mice (53, 54), and data from the majority of studies reveal higher levels of activity in females relative to males (54–56). Although circulating hormones in adulthood can affect these behaviors, differential exposure to gonadal steroids during the perinatal period plays an important role in the development of sexually dimorphic behaviors in the open field (55). Results of early behavioral studies revealed that neonatal administration of testosterone to female rats resulted in male-like behaviors in the open field (55). Data from later studies showed that male ERKO mice exhibited increased rearing and increased center crossings in the open field relative to wild-type males. These data suggest that ER gene disruption masculinized behavior in the open field or that estrogen action during development is important for masculinization of these behaviors (57). These findings are supported by results of a study in which antisense oligonucleotides were used to knock down ER expression in neonatal females. In this study, females that received the antisense oligonucleotides to ER showed an elevation of female-associated behaviors in the open field (77). Data from another test of anxiety and emotionality, the elevated plus maze, have revealed that neonatal castration of male rats results in female-like patterns of behavior, further suggesting a role for perinatal exposure to gonadal steroids in sexually dimorphic behaviors in a novel environment (78).

The behavioral data in the open field reported here are consistent with the idea that perinatal exposure to BPA may act to alter brain development and brain sexual differentiation. As reported, at 27–29 d of age, some behavioral parameters in the open field revealed significant sex differences in offspring born to control mothers that were not seen in offspring born to mothers treated with 250 ng BPA/kg BW/d. Measurements of rearing behavior suggest that BPA exposure may have masculinized this behavior in the prepubertal female mice. Behavioral measurements in 6- to 9-wk-old animals also revealed sex differences in control offspring that were not observed in offspring born to mothers treated with 25 or 250 ng BPA/kg BW/d, indicating that the behavioral differences persist beyond weaning. Although the differences were not statistically significant, animals in the older age group showed a trend consistent with masculinization of rearing behavior in the BPA-exposed females.

No attempt was made to monitor estrous cycles in these young females or to assess gonadal steroid levels in either sex; however, a previous study in our lab found no signifi-
significant effects of perinatal BPA exposure on estradiol levels on the day of the first proestrus (19). As mentioned previously, vaginal smear records of 6- to 9-wk-old females in our colony do not reveal regular patterns of 4- to 5-d estrous cycles, which is probably attributed, in part, to the absence of male pheromones and the presence of female cagemates (reviewed in Ref. 59). Although we cannot rule out the possibility that differences in circulating hormone levels may have influenced behavioral measures in the older animals, it is unlikely that they could solely account for the loss of sex differences in the BPA-exposed offspring; behavioral tests in prepubertal animals also revealed significant sex differences in control offspring that were not observed in BPA-exposed offspring. Given the evidence that neonatal estrogen action through ERs may be important for expression of sexually dimorphic behaviors in the open field, it is possible that the estrogenic actions of BPA are responsible for some of the changes in the behavioral parameters measured.

To date, behavioral studies have examined offspring exposed to significantly higher levels of BPA than those used here (greater than 100-fold), and alterations of both sex-dependent and sex-independent behaviors have been reported in rats (25, 26, 28, 30, 31). Data from these studies reveal the ability of perinatal exposure to BPA to exert complex, multifaceted developmental effects on behavior in adulthood, and not all of the effects described can be attributed to BPA’s action as an estrogen. It is possible that higher levels of BPA exposure can exert different or additional effects on behavioral development. Dose-response curves for estrogenic compounds, including BPA, are complex and nonmonotonic for some endpoints (79).

The importance of protecting the developing female brain from circulating estrogens

Afp has been identified in many vertebrate species. It is abundant during fetal life and decreases in abundance after birth (41, 42). In rodents, Afp binds estradiol with high affinity and high capacity. Recent data from studies of Afp knockout mice (Afp−/−) lend support to the idea that Afp protects the developing female mouse brain from masculinization and defeminization by binding circulating estradiol that may reach the female fetus from the mother or from neighboring male littersmates (40). Of particular relevance to the studies presented here, the number of TH neurons in the AVPV of Afp−/− females was significantly reduced relative to wild-type females and did not differ from wild-type males. Also relevant was evidence of masculinization and defeminization of mating behaviors in the Afp−/− animals. Treatment of the pregnant dams with an aromatase inhibitor to decrease estradiol levels during pregnancy prevented the masculinization of TH neuron number and behavior in Afp−/− females. These data indicate the importance of protecting specific regions of the female mouse brain from estrogen during early development. Relative to estradiol, BPA shows a lower affinity for plasma binding proteins (43), including Afp, and therefore Afp would be expected to provide little protection in shielding areas of the developing brain from exposure to nonsteroidal estrogens like BPA. As a result, BPA and other environmental estrogens might be able to enter regions of the developing female brain that are normally protected from excessive estrogen exposure, and once there, they could alter the expected developmental plan.

Levels of BPA exposure in our animals

In the present study, pregnant mice were implanted with osmotic pumps that chronically released BPA at doses of 25 or 250 ng BPA/kg BW/d. To our knowledge, the BPA exposures used in this study are among the lowest examined to date, and the lowest doses that have shown significant effects after perinatal administration. Data from a recent study that used the very conservative assumption that levels of BPA in urine represent the total amount ingested (7) led to estimates of a maximum daily intake of 0.23 μg BPA/kg BW in the individuals studied. This dose is similar to the high BPA dose used in our experiments. BPA has been shown to cross the placental barrier in mice and rats (15, 80, 81). Although we do not know the precise dose of BPA reaching the developing fetuses in our studies, Zalko et al. (80) reported that 24 h after a single sc injection of 25 μg tritiated BPA/kg BW to pregnant mice on d 17 of gestation, approximately 0.4% of the radioactivity administered to the dams was recovered in the uterus (corresponding to ~3.45 ng/g [3H]BPA equivalents), 0.3% in the amniotic fluid (~4.85 ng/ml [3H]BPA equivalents), 0.6% in the placenta (~3.14 ng/g [3H]BPA equivalents), and 4.1% was present in the entire litter of fetuses (1.23 ng/g [3H]BPA equivalents per fetus). These estimates were obtained after a single acute injection of a 100- to 1000-fold higher dose of BPA than the daily doses chronically administered in the present study. Therefore it is not possible to extrapolate the levels of BPA reaching our animals from these measurements. However, it is interesting to note that the levels estimated in the Zalko study (80) appear to fall within the range of BPA exposure that has been reported in the human fetal-placental unit: 8.3 ng/ml in amniotic fluid at 15–18 wk of gestation, 0.3–18.9 ng/ml in maternal plasma, 0.2–9.2 ng/ml in fetal plasma, and 1.0–104 ng/g in the placenta (10, 11, 82). After parturition, neonatal animals in our study continued to receive exposure to BPA via lactation. Although no measurements are yet available in mice, data from a study in rats showed that 0.003% of an initial high dose of radiolabeled BPA (100 mg BPA/kg BW) was recovered in the lactating dam’s milk 1 h after administration (corresponding to ~1.0 μg equivalent/ml) (81). Recent assessments of BPA in human breast milk have revealed mean levels of 0.61 ng BPA/ml (range, 0.28–0.97 ng/ml) in samples from 23 lactating mothers (12).

Summary

Exposure of pregnant female mice to low levels of BPA from the evening of d 8 of pregnancy through d 16 of lactation results in lasting effects on the brain of the offspring. The data presented reveal alterations in sexually dimorphic anatomical and behavioral endpoints assessed in offspring of mothers exposed to BPA. Although the mechanisms involved in BPA’s actions on the developing brain cannot be delineated from the data presented, we have hypothesized that some of the observed effects may be related to the estrogenic activity
of BPA. Data from studies of ERKO mice have suggested a role for estrogen action during development in the masculinization of TH neuron number (52) and open-field behaviors (57). However, it must be recognized that in addition to its well-documented estrogenicity, BPA may exert other effects on the developing brain. Because of the paucity of available information, it would be premature to speculate about the potential role of putative nonestrogenic effects of BPA at this time. The fact that exposure to low environmentally relevant levels of BPA results in measurable effects should be of considerable concern, particularly if one considers that BPA represents only one of many potential endocrine disruptors in the environment to which humans may be exposed daily.

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References


33. Greco TL, Payne AH 1994 Ontogeny of expression of the genes for steroido-genic enzymes P450 side-chain cleavage, 3α-hydroxysteroid dehydrogenase,
Rubin et al. • BPA Alters Brain Sexual Differentiation

Endocrinology, August 2006, 147(8):3681–3691 3691


53. Le WW, Berghorn KA, Rasmussen E, Hoffman GE 1999 Periventricular preoptic area neurons coactivated with luteinizing hormone (LH)-releasing hormone (LHRH) neurons at the time of the LH surge are LHRH affrents. Endocrinology 140:510–519.


