Organizational Effects of Perinatal Exposure to Bisphenol-A and Diethylstilbestrol on Arcuate Nucleus Circuitry Controlling Food Intake and Energy Expenditure in Male and Female CD-1 Mice

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The endocrine disrupting compound bisphenol-A (BPA) has been reported to act as an obesogen in rodents exposed perinatally. In this study, we investigated the effects of early-life BPA exposure on adult metabolic phenotype and hypothalamic energy balance circuitry. Pregnant and lactating CD-1 dams were exposed, via specially prepared diets, to 2 environmentally relevant doses of BPA. Dams consumed an average of 0.19 and 3.49 μg/kg per day of BPA in the low and high BPA treatments prenatally and an average of 0.36 and 7.2 μg/kg per day of BPA postnatally. Offspring were weaned initially onto a normal (AIN93G) diet, then as adults exposed to either a normal or high-fat diet (HFD). Males exposed to the high dose of BPA showed impaired glucose tolerance on both diets. They also showed reduced proopiomelanocortin fiber innervation into the paraventricular nucleus of the hypothalamus, and when exposed to HFD, they demonstrated increased neuropeptide Y and Agouti-related peptide expression in the arcuate nucleus (ARC). Females exposed to the high BPA dose were heavier, ate more, and had increased adiposity and leptin concentrations with reduced proopiomelanocortin mRNA expression in the ARC when consuming a HFD. BPA-exposed females showed ARC estrogen receptor α expression patterns similar to those seen in males, suggesting a masculinizing effect of BPA. These results demonstrate that early-life exposure to the obesogen BPA leads to sexually dimorphic alterations in the structure of hypothalamic energy balance circuitry, leading to increased vulnerability for developing diet-induced obesity and metabolic impairments, such as glucose intolerance. **(*Endocrinology* 154: 1465–1475, 2013)**

Bisphenol-A (BPA) is a plastic monomer and plasticizer that is used in the production of polycarbonate plastics and epoxy resins. Owing to its structural similarity with endogenous estrogens, such as 17β-estradiol, and potent xenoestrogens, such as diethylstilbestrol (DES), BPA has been described as a potential endocrine disrupting compound (1, 2). Indeed, BPA acts as an agonist to both classical (3) and membrane-bound estrogen receptors (ERs), such as G-coupled protein receptor 30 (4, 5). Because BPA is readily released from many plastic food and drink containers, the effect of low-dose oral exposure to human populations is of great concern (2).

Early risk-assessment studies identified 50 mg/kg per day as the lowest observed adverse effect level; the Environmental Protection Agency reference dose of 50 μg/kg per day was therefore determined using a 1000-fold safety margin (6). Nevertheless, it is becoming evident that BPA may alter the function of a number of organs and tissues, including the brain at levels that are lower than this reference dose (1). Of particular interest here is the possibility that BPA exposure may lead to obesity and metabolic dysfunction. This is supported by studies showing that acute exposure to ecologically relevant doses of BPA impairs insulin signaling in both adult male and pregnant mice (7,

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Abbreviations: AgRP, Agouti-related peptide; ARC, arcuate nucleus; BAT, brown adipose tissue; BPA, bisphenol-A; CON, control; DES, diethylstilbestrol; DIO, diet-induced obesity; ER, estrogen receptor; GTT, glucose tolerance test; HBPA, high BPA; HFD, high-fat diet; LSD, least significant difference; ObRb, leptin receptor long form; PND, postnatal day; POMC, proopiomelanocortin; PVN, paraventricular nucleus; qRT-PCR, quantitative RT-PCR; SOCS3, suppressor of cytokine signaling 3; VCO_2, carbon dioxide production; vGlut, vesicular glutamate transporter 1; VO_2, oxygen consumption.

8). Early-life exposure to low doses of BPA has an obesogenic effect on adult offspring (9–11). These effects have largely been explored in terms of their peripheral mechanisms, including dysfunctions in the form and function of pancreatic β-cells (11) and increases in adipogenic gene expression in white adipose tissue (12).

Within the brain, neurons in the hypothalamic arcuate nucleus (ARC) are critical for the regulation of body weight and energy balance. Neurons in the ARC that synthesize proopiomelanocortin (POMC) are critical for inhibiting feeding and increasing metabolic rate, whereas neurons that produce Agouti-related peptide (AgRP) promote feeding and reduce metabolic rate, acting at the same terminal regions as POMC cells (13, 14). These 2 sets of neurons form the hypothalamic melanocortin system, the activity of which is regulated by hormones such as estradiol (15, 16), leptin (17, 18), and ghrelin (13). Fibers arising from POMC cells innervate downstream structures such as the hypothalamic paraventricular nucleus (PVN), and these projections are critical in coordinating downstream responses to metabolic hormones. In the absence of leptin, this pathway fails to develop, and the adult response to leptin is thereby impaired (19).

The physiological systems regulating feeding and energy balance are sexually differentiated (20, 21). Indeed, the adult function of the melanocortin system shows substantial sex differences, with females having increased responsiveness to the anorectic effects of leptin and decreased responsiveness to the anorectic effects of insulin (20, 21). In adulthood, these differences are known to be estrogen dependent (22, 23). Emerging evidence points to an organizational role for sex steroids in the development of this dimorphism: females exposed perinatally to estradiol showed impairments in the adult anorectic response to leptin and developed masculinized energy balance circuitry (24).

In the current study, we hypothesized that BPA exposure early in life alters the development of the melanocortin system. Furthermore, because early-life estrogen exposure tends to masculinize female energy balance circuitry (24), we hypothesized that the obesogenic effect of exposure to the estrogenic compounds BPA and DES would occur as a result of masculinization of the melanocortin system.

Materials and Methods

Animals

All procedures were approved by the Carleton University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. Virgin female CD-1 mice (n = 20 for cohort 1, n = 16 for cohort 2) were purchased from Charles River (St.-Constant, Canada) and housed in polypropylene cages with ad libitum access to purified AIN93G control diet (Research Diets, New Brunswick, New Jersey) and tap water in glass bottles. Animals were maintained on a 12-hour light, 12-hour dark cycle (lights on at 8 AM). Female mice were weight matched and assigned into 4 treatments (n = 5 for cohort 1, n = 4 for cohort 2). These females were housed in pairs along with a male mouse. AIN93G (Research Diets) was used as the control diet, as well as the basis for the 3 treatment diets. BPA or DES (>99% pure; Sigma-Aldrich, St. Louis, Missouri) was incorporated into AIN93G as described in Ref. 25.

Mated females were inspected every morning for evidence of a vaginal plug, the presence of which marked gestational day 0. Confirmed pregnant females were single housed and placed immediately on 1 of 4 specially prepared diets: 1) AIN93G control diet (control), 2) AIN93G with 1-μg/kg diet BPA (low BPA), 3) AIN93G with 20-μg/kg diet BPA (high BPA), 4) and AIN93G with 4-μg/kg diet DES. DES is a well-characterized ER ligand and is included here as a positive control (25, 26). Dams were maintained on these diets throughout pregnancy and nursing. Food intake and body weight was recorded daily in dams.

Weaning and early life

Litters were adjusted on postnatal day (PND)2 to have 10–12 pups per litter with equal sex ratios where possible. On PND21, pups were weaned and housed in groups of 2–4 according to sex and litter and maintained on the control diet. At PND90, 4 males and 4 females were randomly chosen from each of the litters in cohort 1 to continue in the long-term study, all animals were single housed (n = 160 for cohort 1, n = 128 for cohort 2).

Estrous cycle monitoring

The estrous cycle was monitored by vaginal lavage in female offspring for at least 8 days before any experimental manipulation or measurement. Experiments were carried out irrespective of estrous cycle; however, proestrous animals were removed from subsequent analysis, because this phase of the estrous cycle is associated with altered feeding behavior and neuropeptide expression (27).

Diet-induced obesity (DIO)

At 3 months of age, a subset of animals from cohort 1 (2 males and 2 females from each litter) was placed on a high-fat diet (HFD) (60% of kilocalories from fat, 5.24 kcal/g; Research Diets) to explore their propensity to DIO. Remaining animals continued on the AIN93G control diet (15.8% of kilocalories from fat, 3.9 kcal/g) throughout the experiment.

Indirect calorimetry

Analyses of whole-body energy expenditure were performed on animals in cohort 1 at 3 months of age (before HFD) and again at 5 months of age (after HFD) using an open-circuit indirect calorimetry system (LabMaster, TSE Systems, Chesterfield, Missouri). Oxygen consumption (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio, and locomotion were measured. Energy expenditure was calculated using the following equation: $\text{kcal/h} = (3.941 \times VO_2 + 1.106 \times VCO_2)/1000$. Animals were given 24 hours to habituate to the chambers, followed immediately by 24 hours of recording.

Glucose tolerance tests (GTTs)

GTTs were performed in the morning (9 AM) after approximately 60 days of HFD exposure as described in Ref. 7. Mice

were excluded from analysis if they did not exhibit an increase of at least 1 mmol/L 15 minutes after injection.

Plasma analysis

Mice in cohort 1 were killed at 5.5 months of age by decapitation. Trunk blood was collected at the time of killing. Plasma concentration of the adipokines IL-6, insulin, leptin, and resistin were measured simultaneously using MilliPlex Mouse Adipokine kits (Millipore, Bedford, Massachusetts) and a Luminex 100 analyzer (Luminex, Billerica, Massachusetts).

Body fat determination

Carcasses of animals from cohort 1 were frozen at −20°C before dissection. Perigonadal, retroperitoneal, sc fat pads, and interscapular brown adipose tissue (BAT) were dissected and weighed by an observer blind to treatment condition.

Histology

Animals from cohort 2 were killed at 3 months of age by transcardial perfusion with 0.9% saline, followed by 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose and sectioned at 60 μm on a cryostrat in a 1-in-4 series. Pooled data from 2 brains per litter per sex were analyzed; brains with insufficient numbers of anatomically matched sections were excluded from analysis (final n = 3–4).

Double immunohistochemistry for ERα and POMC

Free-floating tissue sections were subjected to double immunohistochemistry according to our lab's standard protocols (28) using monoclonal mouse anti-ERα antibody (1:500; DAKO, Glostrup, Denmark) for 72 hours at 4°C followed by rabbit anti-POMC antibody (1:10,000; Phoenix Pharmaceuticals, Burlingame, California) for 24 hours at room temperature.

Immunofluorescence for vesicular glutamate transporter 1 (vGlut) or glutamic acid decarboxylase 67 (GAD67) and POMC, and AgRP

Tissue sections were processed for either vGlut1 and POMC, or GAD67 and POMC immunofluorescence in a manner similar to previously published (29). Primary antibody cocktails contained rabbit anti-POMC (1:2500; Phoenix Pharmaceuticals) and either mouse anti-vGlut1 (1:1000; Millipore) and/or mouse anti-GAD67 (1:500; Millipore). Sections stained for AgRP were processed as above with goat anti-AgRP (1:1000; Santa Cruz Biotechnology, Inc, Santa Cruz, California) and donkey antigoat Alexa Fluor 594 IgG (1:200; Life Technologies, Carlsbad, California).

Stereology

Brains double stained for ERα and POMC were analyzed using unbiased stereology to estimate the total number of ERα-, POMC-, and double-stained cells in the ARC. The ARC was outlined bilaterally at low magnification (×4) through sections encompassing the anterior and posterior extent of the nucleus using StereoInvestigator Software (MicroBrightField, Williston, Vermont). The optical fractionator method was used to estimate the total number of cells in the region of interest at high magnification (×60). Dissector frames of 80 μm^2 were placed along a 100-μm grid within each contour. The dissector height was set at 15 μm. Counts were estimated by StereoInvestigator software.

Quantification of vGlut1 and GAD67 putative synaptic contacts

Sections were analyzed at ×100 magnification using an Olympus BX61 microscope equipped with an Olympus DSU disk-scanning confocal unit (Olympus, New York, New York). POMC cell perimeter, vGlut1, and GAD67 containing puncta near POMC perikarya were quantified in at least 10 POMC cells each per animal.

Quantification of POMC and AgRP projections

Z-stacks at 1-μm intervals through the PVN were collected at low power (×10) using an Olympus BX61 microscope equipped with an Olympus DSU disk-scanning unit confocal unit. Image Pro Analyzer (Media Cybernetics, Rockville, Maryland) was used to collapse z-stacks into composite images. Composite images were then binarized and skeletonized using identical parameters for each image. Staining intensity was expressed as a percent of that observed in the same-sex control animals.

Quantitative RT-PCR (qRT-PCR)

One animal per sex and postnatal diet was randomly selected from each litter in cohort 1 for analysis of gene expression using qRT-PCR, except where prohibited by attrition or estrous status, yielding n = 3–5 animals per sex, treatment, and diet condition. Micropunches of the ARC were homogenized, and RNA was isolated in TRIzol (Life Technologies) and reverse transcribed using the SuperScript II kit with oligo(DT) and the method provided by the manufacturer (Life Technologies). Quantitative real-time PCR was performed in a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, California) using iQ SYBR Green Super Mix (Bio-Rad). Gene expression was determined relative to the housekeeping control genes glyceraldehyde 3-phosphate dehydrogenase and β-actin using the $2^{-\Delta\Delta Ct}$ method (30). Control (CON) males consuming standard chow were used as the control group for all qRT-PCR analyses. All primer pairs were efficient over 5 orders of magnitude. Primer sequences are found in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Statistics

To avoid confounds due to litter effects, littermates of the same sex and postnatal diet were pooled in all statistical analyses as recommended by Ref. 31, yielding n = 5 and n = 4 for cohorts 1 and 2, respectively. Animals that developed home-cage stereotypies (excessive grooming, flipping) were excluded from analysis. Data are expressed in graphs as mean ± SEM. Data were analyzed using 2-way ANOVA with sex and perinatal treatment as factors. Where applicable, diet was included as an additional factor. Metabolic data were also analyzed using a repeated-measures ANOVA to compare pre-HFD and post-HFD parameters. Significant main or interaction effects were further analyzed using Fisher's protected least significant difference (LSD) with a significance criterion set at $P < .05$.

Results

BPA exposure does not affect maternal body weight or food intake

Exposure to BPA or DES did not differentially affect maternal food intake or body weight at any point pre- or

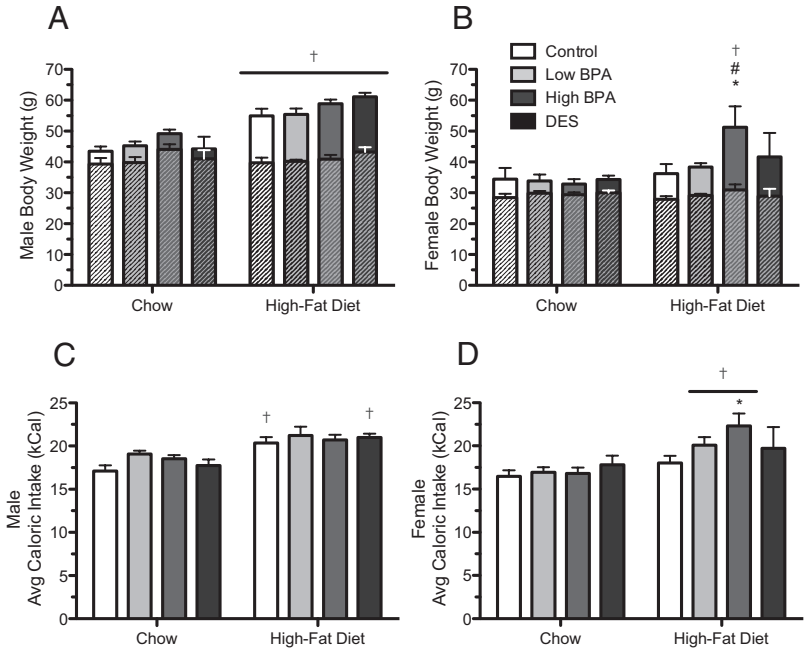

Figure 1. Weight of chow and HFD mice at 3 (overlaid) and 5.5 months of age after HFD exposure, in males (A) and females (B). Average daily caloric intake of male (C) and female (D) mice during the period of HFD exposure; n = 3–5 per sex per treatment per diet condition. Data are expressed as mean ± SEM. Symbols indicate significant Fisher's LSD post hoc comparisons: $*P < .05$ vs CON of the same sex and diet; $\#P < .05$ vs DES positive control of the same sex and diet; $\dagger P < .05$ vs animals of the same treatment and sex consuming chow.

postnatally (Supplemental Tables 2 and 3). Pregnant dams consumed an average of 0.19 and 3.49 μg/kg per day of BPA in the low and high BPA treatments, respectively. During PND0–PND13, dams consumed an average of 0.36 and 7.2 μg/kg per day of BPA in the low and high BPA treatments (Supplemental Table 4). Dams in the DES treatment consumed an average of 0.64 μg/kg per day during pregnancy and 1.41 μg/kg per day during lactation (Supplemental Table 4). Because pups begin reaching for solid food as of PND14, data subsequent to these days do not reflect maternal dose alone.

BPA-exposed females eat more and gain more weight when fed a HFD

Maternal treatment did not affect body weight in either male or female offspring at 3 months of age (Figure 1, A and B). After HFD exposure, all male mice showed significant increases in body weight as compared with males who remained on regular chow (Figure 1A), but neither weight nor food intake (Figure 1C) was differentially affected by maternal treatment. When consuming the HFD, females perinatally exposed to the high dose of BPA were heavier than female controls and females that were exposed to DES (Figure 1B). This increase in body weight was accompanied by a significant increase in caloric intake relative to control females consuming HFD (Figure 1D).

In spite of not showing differences in body weight from controls, chow-consuming males exposed to BPA at the high dose exhibited increased weight in the retroperitoneal and intrascapular brown adipose fat pads compared with control and DES-exposed mice (Figure 2A). When given the HFD, male mice exposed to both doses of BPA or DES had larger retroperitoneal fat pads than control mice. There was no effect of maternal treatment on any of the fat pads among females consuming chow (Figure 2B). Female mice exposed to the higher dose of BPA showed significantly increased perigonadal, retroperitoneal, BAT, and sc fat pad weights compared with control females also receiving the HFD, and larger perigonadal and BAT fat pads than females exposed to DES also eating the HFD (Figure 2D).

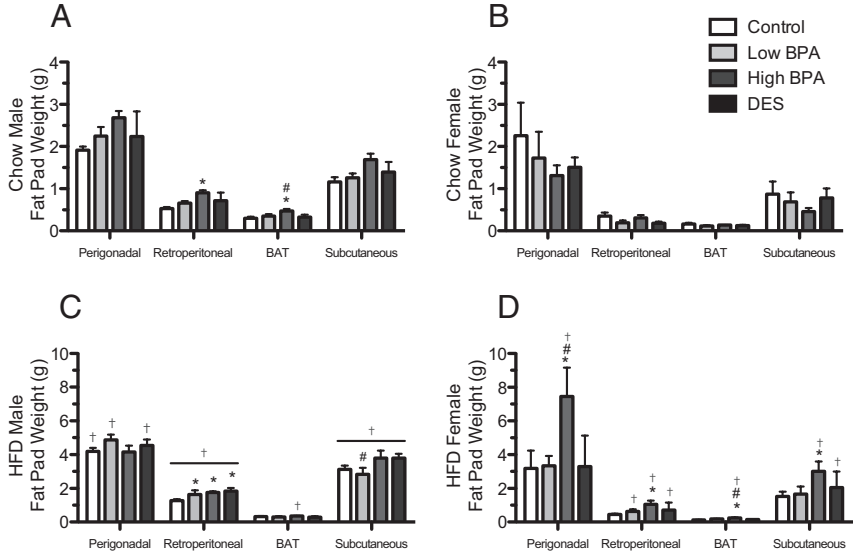

Figure 2. Postmortem weight of fat pads in male (A and C) and female (B and D) mice consuming chow (A and B) and HFD (C and D); n = 3–5 per sex per treatment per diet condition. Data are expressed as mean ± SEM. Symbols indicate significant Fisher's LSD post hoc comparisons: $*P < .05$ vs CON of the same sex and diet; $\#P < .05$ vs DES positive control of the same sex and diet; $\dagger P < .05$ vs animals of the same treatment and sex consuming chow.

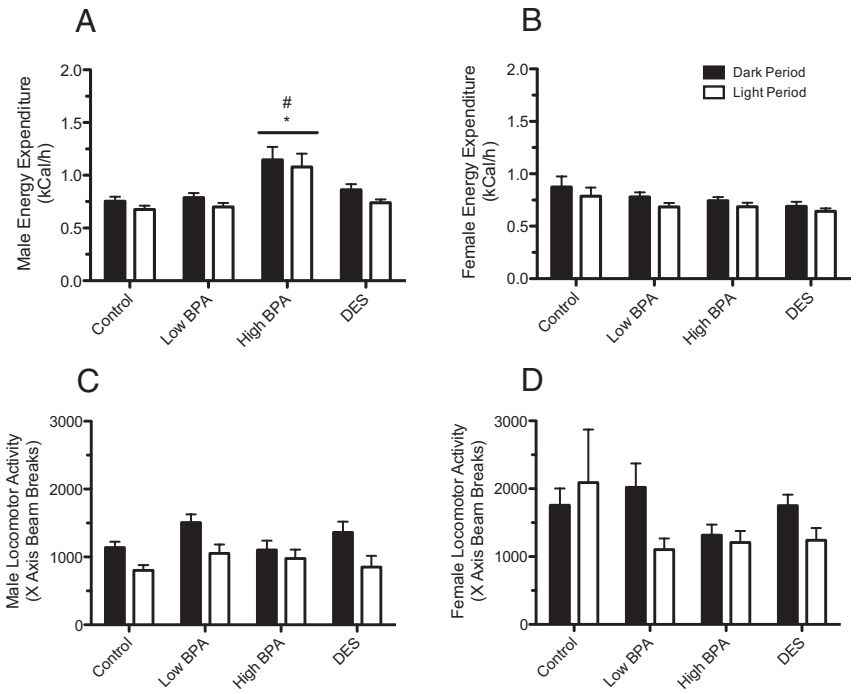

Figure 3. Energy expenditure measured in males (A) and females (B) at 3 months of age, before HFD exposure. Cumulative 24-hour locomotor activity in males (C) and females (D) measured in mice during metabolic phenotyping. Dark bars indicate measures collected during the dark period of the light-dark cycle (8 PM to 8 AM); n = 4–5 per sex per treatment condition. Data are expressed as mean ± SEM. Symbols indicate significant Fisher's LSD post hoc comparisons: $*P < .05$ vs CON of the same sex; $\#P < .05$ vs DES positive control of the same sex.

BPA exposure affects energy expenditure in males

Before being exposed the HFD, male mice that were exposed to the high dose of BPA showed significantly elevated energy expenditure at 3 months of age as compared with control and DES-treated males, (Figure 3A). This was not accompanied by any changes in locomotor activity (Figure 3C). Female mice did not show any treatment-related metabolic or locomotor alterations at this point (Figure 3, B and D). At 5 months of age, energy expenditure in chow-consuming male mice exposed to the high dose of BPA was still higher than control, low BPA- or DES-treated male mice (Figure 4A). When exposed to HFD, high-dose BPA males showed significantly reduced energy expenditure compared with their own pre-HFD time point (Figure 4A). This could not be accounted for by an alteration in locomotor activity, because there were no treatment effects seen in this measure (Figure 4C). Females did not show any differences in energy expenditure (Figure 4B), although light period locomotion among HFD-consuming female mice exposed the high BPA dose was significantly reduced compared with their chow-consuming counterparts (Figure 4D). VO_2 and VCO_2 are used directly in calculating energy expenditure, and therefore, their trends largely mirror those reported in energy expenditure (Supplemental Figures 1, A–D, and 2, A–D).

Males exposed to BPA show impaired glucose tolerance

Males exposed to the high BPA dose showed significant impairments in glucose tolerance regardless of the diet they were being fed (Figure 5, A, C, and E). This was particularly evident when data were expressed as area under the curve, with both chow- and HFD-consuming males showing impaired glucose tolerance relative to their respective controls (Figure 5A). There were no treatment-related impairments in glucose tolerance observed among females consuming either chow or HFD (Figure 5B), although control female mice consuming chow showed significantly elevated blood glucose at 15 minutes after injection compared with BPA- or DES-treated female mice (Figure 5D). This effect was not seen in females consuming HFD (Figure 5F).

Endocrine effects of early-life exposure to BPA

HFD exposure had the expected effect of increasing plasma leptin in both males and females, excluding female DES-exposed mice. There were no significant treatment-related differences in plasma leptin concentrations observed in males. Female mice exposed to the higher dose of BPA and consuming HFD showed increased plasma leptin concentrations compared with control female mice also fed HFD (Supplemental Table 5). In contrast, DES female mice consuming HFD showed decreased circulating leptin compared with HFD-fed control female mice (Supplemental Table 5). Male mice exposed to the high dose of BPA or DES showed significant increases in plasma insulin while eating HFD compared with HFD-fed control mice and with their chow-consuming counterparts (Supplemental Table 5). Circulating levels of resistin were also altered by exposure to BPA. For instance, resistin levels in HFD-fed male mice exposed to the low dose of BPA were higher than those of high fat-fed controls. Females exposed to the high BPA dose had higher plasma resistin levels when consuming HFD compared with both their control and DES-treated female mice. There were no significant alterations in circulating IL-6 in any of the treatments (Supplemental Table 5).

Effects of BPA on POMC and AgRP innervation of the PVN

Immunocytochemical examination of cohort 2 mice at 3 months of age revealed that males exposed to higher dose

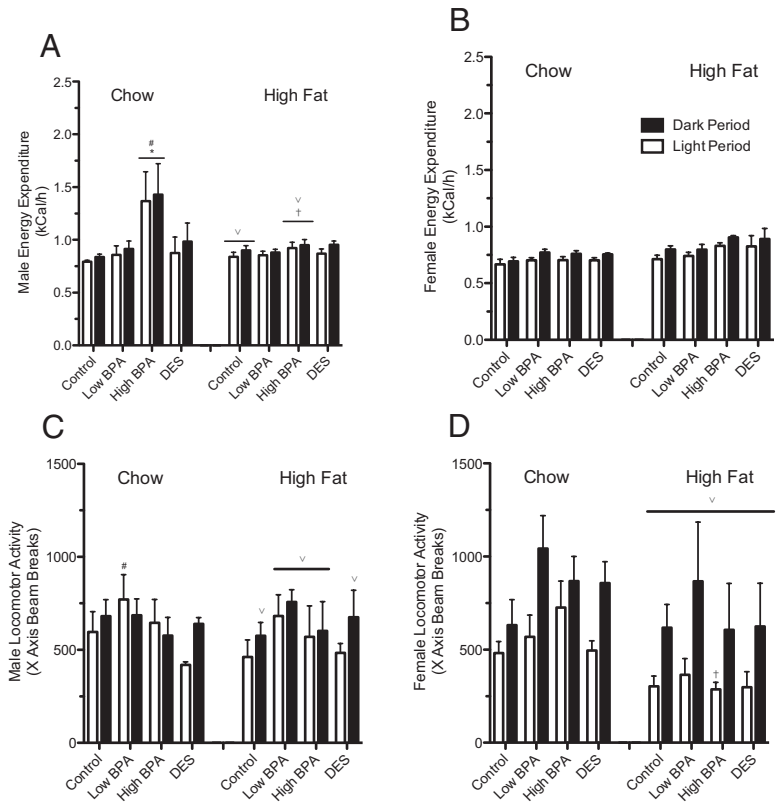

Figure 4. Energy expenditure measured in males (A) and females (B) at 5 months of age, after HFD exposure. Cumulative 24-hour locomotor activity in males (C) and females (D) measured in mice during metabolic phenotyping. Dark bars indicate measures collected during the dark period of the light-dark cycle (8 PM to 8 AM); n = 3–5 per sex treatment diet condition. Data are expressed as mean ± SEM. Symbols indicate significant Fisher's LSD post hoc comparisons: $*P < .05$ vs CON of the same sex and diet; $\#P < .05$ vs DES positive control of the same sex and diet; $\dagger P < .05$ vs animals of the same treatment and sex consuming chow; $vP < .05$ repeated measures ANOVA vs data collected before HFD exposure.

of BPA showed significantly decreased POMC fiber density in the PVN relative to control mice and DES-treated mice (Figure 6A). In females, DES resulted in a similar decrease in fiber density as compared with control females (Figure 6A). There were no effects of BPA or DES treatment on AgRP fiber innervation of the PVN of male mice. In females, however, DES treatment resulted in increased AgRP fiber innervation of the PVN compared with females exposed to the low and high doses of BPA (Figure 6D).

BPA exposure does not affect vGlut and GAD67 inputs onto POMC cells

Perinatal BPA or DES treatment did not affect the number of excitatory, vGlut immunopositive putative contacts onto POMC-expressing cells in the ARC in either sex. There was, however, a significant effect of sex in this measure, with females showing significantly more contacts per 100 μm of perikarya (Figure 6G). There were also no treatment-related differences in the number of inhibitory, GAD67 immunopositive putative contacts onto POMC cells in either sex (Figure 6G).

ERα and POMC counts in the ARC are altered by BPA exposure

No significant treatment-related differences were seen in the total number of ERα immunopositive cells in the ARC in males (Figure 6J). In females, however, early-life exposure to low or high doses of BPA resulted in a significant increase in ERα positive cells in the posterior portion of the ARC relative to control females (Figure 6J). None of the treatments affected the total number of POMC cells neurons in the ARC of male mice. In females, early-life DES treatment resulted in a higher number of POMC immunoreactive cells in the posterior ARC compared with CON females (Figure 6L). Male and female mice exposed to the low dose of BPA had a higher proportion of POMC cells coexpressing ERα in the posterior ARC compared with their respective control mice. A similar effect was produced by exposure to the higher dose of BPA in females (Figure 6N).

BPA exposure affects the transcriptional response to HFD

Gene expression analyses from ARC samples revealed that neither perinatal treatment nor diet affected ERα mRNA expression in male mice (Figure 7A). In female mice, however, exposure to the low dose of BPA resulted in increased ERα mRNA expression in the hypothalamus relative to control and DES-treated females consuming regular chow (Figure 7A). Exposure to HFD resulted in elevated ERα expression among females that were exposed to the high dose of BPA early in life compared with high fat-fed control mice (Figure 7A).

Male mice exposed to the higher dose of BPA expressed significantly higher levels of AgRP mRNA compared with control mice when fed a HFD (Figure 7B). A similar trend was observed when examining neuropeptide Y mRNA expression in these same animals ($P = .07$) (Figure 7C). No treatment-related differences were seen in these transcripts among females consuming either diet.

There were no differences in POMC mRNA expression between male mice in the different experimental groups. In control females, adult exposure to the HFD increased POMC expression in the ARC as compared with their

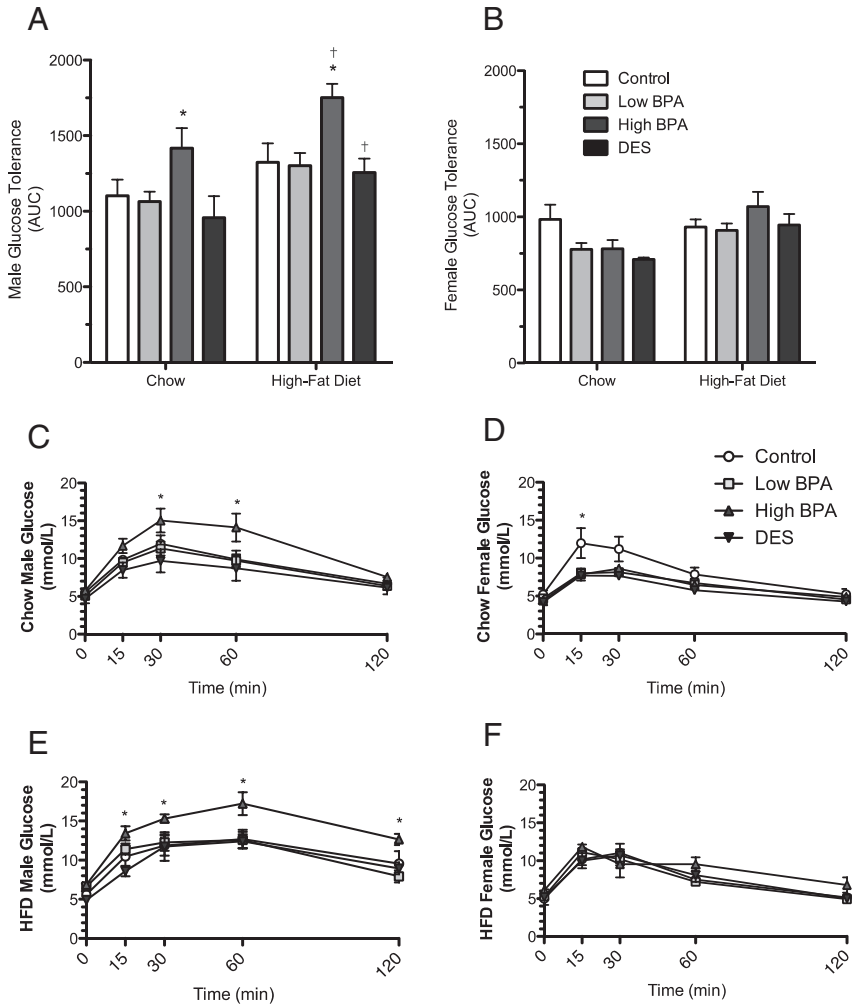

Figure 5. Cumulative GTT results expressed as area under the curve (AUC) in male (A) and female (B) mice at 5 months of age, after HFD exposure. Blood glucose measures at individual time points during the test in males (C and E) and females (D and F) consuming chow (C and D) and HFD (E and F); n = 2–5 per sex per treatment per diet condition. Data are expressed as mean ± SEM. Symbols indicate significant Fisher's LSD post hoc comparisons: $*P < .05$ vs CON of the same sex and diet; $\#P < .05$ vs DES positive control of the same sex and diet; $\dagger P < .05$ vs animals of the same treatment and sex consuming chow.

chow-consuming counterparts. Diet did not significantly elevate POMC expression in females exposed to either dose of BPA or DES (Figure 7D).

There were no significant differences in the expression of leptin receptor long form (ObRb) between experimental groups, with the exception of a trend toward elevated expression ($P = .06$) in chow-fed DES-treated female mice as compared with control females (Supplemental Figure 3A). Chow-consuming females exposed to the higher dose of BPA showed significantly lower ObRb expression than DES females also consuming chow (Supplemental Figure 3A). Suppressor of cytokine signaling 3 (SOCS3) expression was elevated in high BPA (HBPA) males exposed to HFD but only relative to DES males (Supplemental Figure 3B). SOCS3 expression was not altered in female mice. The expression of signal transducer and activator of transcription 3 was significantly higher in HFD-consuming DES-exposed males as compared with control males as well as chow-consuming DES-exposed males (Supplemental Figure 3C). There were no significant differences seen between groups or dietary conditions in the expression of Forkhead box protein O1 (Supplemental Figure 3D).

Discussion

At ecologically relevant doses, BPA is capable of exerting acute effects in adult animals and organizational effects in developing animals. However, a greater interest is usually given to its developmental effects as an endocrine disrupter, given that these effects may persist long beyond the initial period of exposure. BPA is capable of passing through the placental barrier (32) and is found in the milk of dams exposed to oral BPA (33). The fetus and neonate are therefore at risk of exposure during the extent of their maternal dependency, a period encompassing all the major phases of organogenesis, sexual differentiation, and neural development.

We found few metabolic effects among males and females exposed to our lower dose of BPA, findings that confirm those of an earlier study conducted using similar methodology (24). Interestingly, the measures affected by our higher dose of BPA showed many sex differences. Consistent with a recent study carried out in rats (11), our high-dose BPA-exposed male mice showed impaired glucose tolerance on both chow and HFD, although in contrast, we did not find them to gain more weight or body fat than controls on HFD. This highlights the important of considering species and diet when assessing the effects of BPA, because our HFD may have led to a ceiling effect in male mice. These mice also showed significantly elevated energy expenditure and BAT mass only when consuming regular chow, suggesting alterations in thermogenesis abolished by HFD exposure. Females generally resisted the development of DIO, showing only modest gains compared with chow-consuming fe-

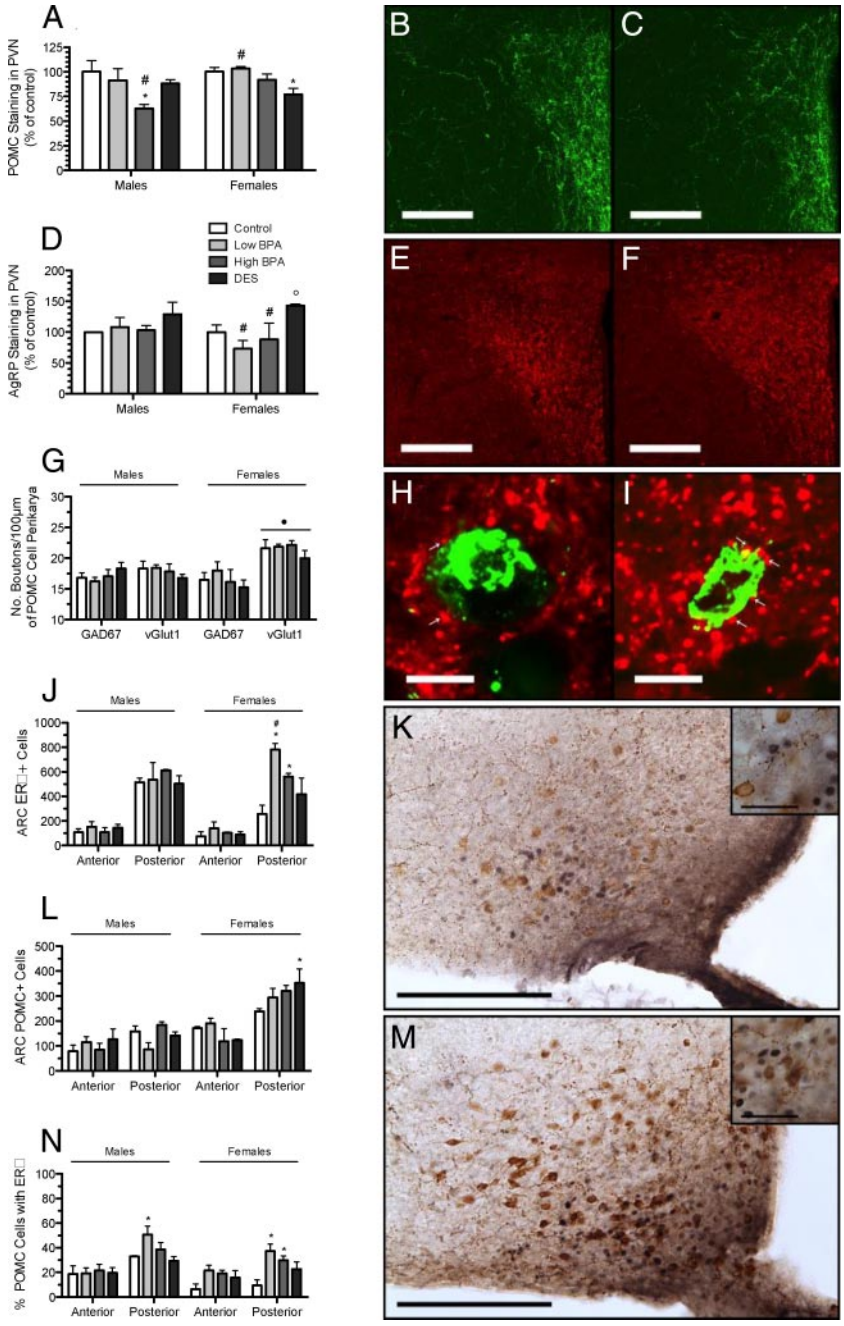

Figure 6. Histological results in 3-month-old male and female mice consuming chow from cohort 2. POMC fiber density in the PVN (A). Representative images of POMC staining from male CON (B) and HBPA (C) animals; scale bars indicate 200 μm. AgRP fiber density in the PVN (D). Representative images of AgRP staining in female CON (E) and DES (F) females; scale bars indicate 200 μm. Fiber density in (A) and (D) are expressed as percent over same-sex CON animals. Number of excitatory vGlut1 and inhibitory GAD67 boutons in contact with POMC cells per 100 μm of perikarya in males and females (G). Representative image of vGlut1 (red) contacts on POMC cells (green) in male (H) and female (I) CON animals; scale bars indicate 10 μm, arrows indicate putative boutons. Stereological estimates of ERα (J) and POMC (L) immunopositive cells in male and female ARC slices. Percent of POMC-expressing cells that coexpress ERα (N). Representative images of female CON (K) and HBPA (M) ARC slices, ERα is revealed as blue nuclear staining, POMC as brown cytoplasmic staining; scale bars indicate 200 μm ($\times$20) and 50 μm ($\times$60, inset); n = 4 per sex per treatment. Data are expressed as mean $\pm$ SEM. Symbols indicate significant Fisher's LSD post hoc comparisons: *$P < .05$ vs CON of the same sex; #$P < .05$ vs DES positive control of the same sex; ●$P < .05$ vs males in same treatment; o$P < .1$ vs CON of the same sex.

males. Females exposed to the higher dose of BPA, like males, responded to HFD with substantial weight and fat gain. These animals did not show signs of impaired glucose tolerance, but this could be because females are generally more glucose tolerant than males, even when important metabolic sensors, such as leptin, are malfunctioning (34). It is conceivable that BPA-exposed female mice did not develop additional metabolic adversity, because the protective effects of central or peripheral estrogen signaling remained intact (35).

Under conditions of energy excess, increases in circulating leptin stimulate POMC expression that in turn organizes a reduction in food intake designed to restore the organism to homeostasis (17, 18). In a state of leptin resistance brought on by protracted HFD exposure, this sequence of events no longer takes place. HFD-exposed males were uniformly hyperleptinemic and did not exhibit an increase in POMC mRNA expression. Females exposed to the higher dose of BPA resembled males in that they were also hyperleptinemic and failed to show an increase in POMC transcription. Because we did not detect any alterations in excitatory vGlut or inhibitory GAD67 contacts onto POMC cells, it is not likely that observed differences in expression were due to alterations in local innervation of POMC neurons (15, 36). To further explore our findings, we analyzed expression of the leptin receptor ObRb, as well as the downstream modulators of leptin signaling signal transducer and activator of transcription 3, SOCS3, and Forkhead box protein O1 (17, 37), although we did not detect any treatment-related differences that could account for the observed results. It is also interesting to note that in male mice exposed to the high dose of BPA, the orexigenic peptides AgRP and neuropeptide Y responded to HFD exposure by showing up-regulation. Future studies will be required to further explore the mecha-

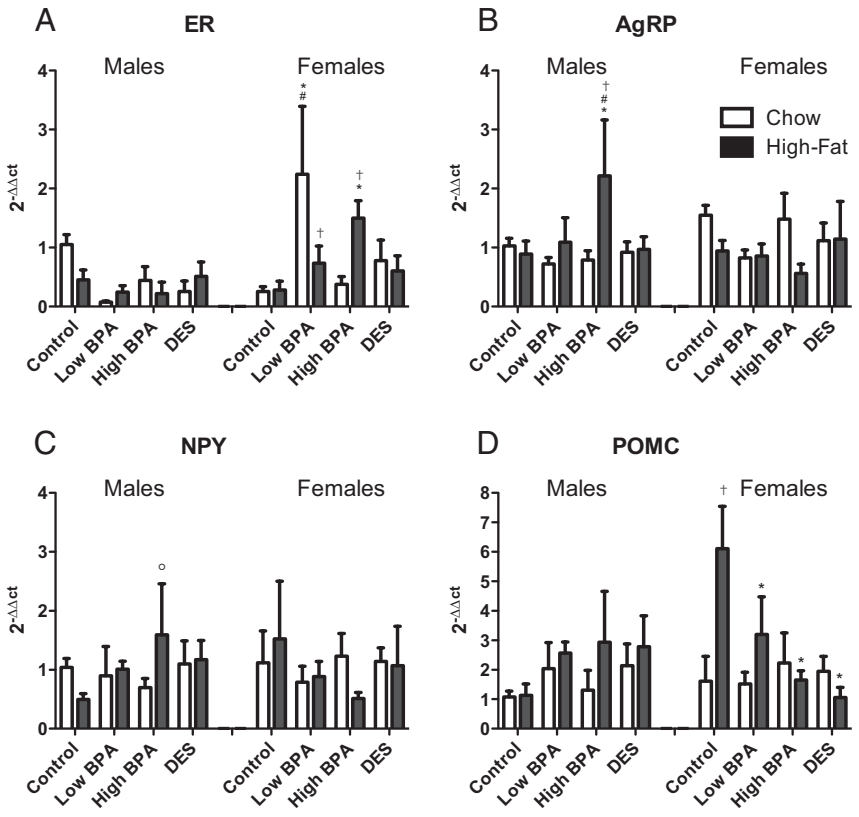

Figure 7. Real-time PCR data from ARC tissue punches in chow- and HFD-consuming male and female mice at 5.5 months of age; n = 3–5 per sex per treatment per diet condition. Data are expressed as mean ± SEM. Symbols indicate significant Fisher's LSD post hoc comparisons: $*P < .05$ vs CON of the same sex and diet; $\#P < .05$ vs DES positive control of the same sex and diet; $\dagger P < .05$ vs animals of the same treatment and sex consuming chow; $oP < .1$ vs CON of the same sex.

nisms that may bring about these transcriptional differences.

We observed several interesting sex differences in our exploration of ERα and POMC expression in the ARC. CON males showed higher expression of ERα and double-labeled cells in the posterior ARC compared with CON females, although in BPA-exposed females, these measures were found to be at levels comparable with, or greater than, seen in our males. This is in agreement with several previous investigations showing that early-life exposure to BPA leads to an increase in ERα expression in a variety of hypothalamic nuclei (38, 39). ERα and POMC double-labeled cells are known to be involved in the anorexigenic effect of the estrogens, including their ability to up-regulate POMC expression (15, 16). These cells are similarly involved in the behavioral and neurobiological effects of leptin and insulin (16). Importantly, the biological effect of leptin appears to synergize with estradiol, such that intact females show a stronger metabolic response to it than intact males (22, 23). These effects are seemingly at odds with the tendency toward DIO and leptin resistance proposed earlier. However, because such effects were seen in both low BPA and HBPA females, POMC/ERα population alone may not be related to the predisposition to DIO. Because effects on ERα expression were not present in DES-exposed animals, their developmental origins are likely not a direct consequence of ER stimulation. Moreover, the population of POMC cells in the ARC is functionally heterogeneous, with certain cells preferentially sensitive to leptin (17) and others to insulin (40). Although we did not evaluate specific cell identity, it is notable that there were no significant differences seen in the anterior ARC, where most of the leptin-responsive POMC cells reside (17).

Exposure to the higher dose of BPA resulted in significantly reduced POMC fiber staining in the PVN in male animals. This response to BPA appeared to be sex specific, because it was not observed in females, although the development of this system may simply be more robust in these animals. DES females were similarly affected, suggesting an ER-mediated mechanism, although the origin of the sex difference here warrants further study. It is likely that these observations reflect postnatal disruption of axonal migration, because this pathway is known to develop under the control of leptin during the early postnatal period (19). These findings are not likely to arise solely from a difference in POMC neurons, because stereological investigation of the ARC did not reveal a decrease in the number of POMC immunoreactive neurons in these groups, nor was there a difference in adult POMC mRNA expression.

In our hands, DES-exposed mice did not show any particular propensity toward DIO. This is in contrast to several published reports demonstrating obesity in mice perinatally exposed to 1 and 10 μg/kg per day doses of DES (41, 42). Differences in postnatal treatment, mouse strain (42), dosing method, and regime (41) all limit direct commensurability between studies. A recent investigation into the topic found that both male and female DES-exposed mice showed reduced body fat under HFD (25), and although the experimental methodology employed was very similar to our own, our use of a 60% fat diet as opposed to a 40% fat diet may have concealed such differences in our own animals. Early-life DES exposure is known to exert a masculinizing influence on the female brain, enlarging the sexually dimorphic nucleus of the preoptic area toward a more male-typical size (43, 44). Although we

found sex differences in ERα and POMC expression in the ARC, these did not appear to be reversed by our DES treatment. The ontogeny of these differences is not known and may depend on hormonally mediated control of apoptosis, as seen in the development of the sexually dimorphic nucleus of the preoptic area and anteroventral periventricular nucleus (45), or it may depend on control of cellular phenotype. Further study into the normal development of these sex differences will be needed to frame our understanding of the effects of the endocrine disruptors employed here. Although DES is acknowledged to be a valid positive control for developmental studies of BPA exposure (46), there exists no satisfactory method for comparing the relative potencies of these chemicals in vivo, and thus, determining a dose optimal for a positive control remains difficult. Nevertheless, brains from our DES-exposed females did show a variety of structural and functional changes, many of which did not overlap substantially with those seen in BPA-exposed animals. These findings are important in differentiating between effects resulting primarily from ER stimulation caused by DES and those of putatively mixed origin caused by BPA.

There exists a certain degree of controversy regarding feed selection in studies of this sort. As highlighted earlier, the potential of ceiling effects associated with the use of extremely HFDs such as ours are an ever-present risk. Our offspring were weaned onto AIN93G, a formulation that possesses a relatively higher fat content than typical maintenance diets. Observations of diet effects, reflecting comparisons between animals consuming a moderate and extremely HFD, must be considered in that light. Moreover, although we elected to use phytoestrogen-free purified chow as the basis of our treatment and control diets, such a strategy is itself known to affect metabolism (47, 48). However, using a purified diet afforded the degree of consistency critical for our model that cannot be easily achieved using standard formulations.

The traditional mode of action for BPA is through stimulation of the classical ERs, including ERα and ERβ (3, 49), and membrane-bound ERs, such as GPR30 (4, 5). Although these mechanisms are certainly relevant, emerging evidence points to an even broader range of possible targets that may be regulated by BPA, including aromatase (50, 51) and the androgen receptor (52). How these targets respond to endocrine disruption over a developmental timescale is not currently well understood. Given the sexually dimorphic nature of the phenotype presented here, the phenotypic differences observed between DES- and BPA-treated animals, and the complexity of BPA pharmacokinetics in vivo, it seems likely that no single target can entirely account for BPA's developmental effects. Further research is required to elaborate on the full scope of targets disrupted by BPA during development.

We have shown here that early-life ecologically relevant BPA exposure leads to a sexually dimorphic adverse metabolic phenotype. Importantly, our doses are well within the realm of the ecologically plausible and well below the Environmental Protection Agency reference dose of 50 μg/kg per day. BPA exerts an organizational effect on the ARC, with females showing patterns of gene expression similar to those seen in control males, and both sexes showing differences in how the circuitry responds to dietary challenge. Because of the ubiquity of BPA in the environment, these findings further the case that exposure to this chemical constitutes a risk factor in the developmental origins of metabolic disease.

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References

1. **Rubin B.** Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. *J Steroid Biochem Mol Biol*. 2011;127:27–61.
2. **Vandenberg L, Hauser R, Marcus M, Olea N, Welshons W.** Human exposure to bisphenol A (BPA). *Reprod Toxicol*. 2007;24:139–216.
3. **Gould J, Leonard L, Maness S, et al.** Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. *Mol Cell Endocrinol*. 1998;142:203–217.
4. **Dong S, Terasaka S, Kiyama R.** Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. *Environ Pollut*. 2011;159:212–220.
5. **Thomas P, Dong J.** Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *J Steroid Biochem Mol Biol*. 2006;102:175–184.
6. **Richter C, Birnbaum L, Farabollini F, et al.** In vivo effects of bisphenol A in laboratory rodent studies. *Reprod Toxicol*. 2007;24:199–423.
7. **Alonso-Magdalena P, Vieira E, Soriano S, et al.** Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring. *Environ Health Perspect*. 2010;118:1243–1250.
8. **Batista TM, Alonso-Magdalena P, Vieira E, et al.** A Short-term treatment with bisphenol-A leads to metabolic abnormalities in adult male mice. *PLoS One*. 2012;7:e33814.
9. **Miyawaki J, Sakayama K, Kato H, Yamamoto H, Masuno H.** Perinatal and postnatal exposure to bisphenol a increases adipose tissue mass and serum cholesterol level in mice. *J Atheroscler Thromb*. 2007;14:245–252.

10. **Rubin BS, Murray MK, Damassa DA, King JC, Soto AM.** Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. *Environ Health Perspect*. 2001;109:675–680.
11. **Wei J, Lin Y, Li Y, et al.** Perinatal exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in adult rats on a high-fat diet. *Endocrinology*. 2011;152:3049–3061.
12. **Somm E, Schwitzgebel VM, Toulotte A, et al.** Perinatal exposure to bisphenol a alters early adipogenesis in the rat. *Environ Health Perspect*. 2009;117:1549–1555.
13. **Abizaid A, Gao Q, Horvath TL.** Thoughts for food: brain mechanisms and peripheral energy balance. *Neuron*. 2006;51:691–702.
14. **Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ.** The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *Int J Obes Relat Metab Disord*. 2001;25(suppl 5):S63–S67.
15. **Gao Q, Mezei G, Nie Y, et al.** Anorectic estrogen mimics leptin's effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals. *Nat Med*. 2007;13:89–94.
16. **Hirosawa M, Minata M, Harada KH, Hitomi T, Krust A, Koizumi A.** Ablation of estrogen receptor α (ERα) prevents upregulation of POMC by leptin and insulin. *Biochem Biophys Res Commun*. 2008; 371:320–323.
17. **Munzberg H, Huo L, Nillni EA, Hollenberg AN, Bjorbaek C.** Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin. *Endocrinology*. 2003;144:2121–2131.
18. **Schwartz MW, Seeley RJ, Woods SC, et al.** Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes*. 1997;46:2119–2123.
19. **Bouret SG, Draper SJ, Simerly RB.** Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science*. 2004;304:108–110.
20. **Shi H, Seeley RJ, Clegg DJ.** Sexual differences in the control of energy homeostasis. *Front Neuroendocrinol*. 2009;30:396–404.
21. **Shi H, Strader AD, Sorrell JE, Chambers JB, Woods SC, Seeley RJ.** Sexually different actions of leptin in proopiomelanocortin neurons to regulate glucose homeostasis. *Am J Physiol Endocrinol Metab*. 2008;294:E630–E639.
22. **Clegg DJ, Riedy CA, Smith KA, Benoit SC, Woods SC.** Differential sensitivity to central leptin and insulin in male and female rats. *Diabetes*. 2003;52:682–687.
23. **Clegg DJ, Brown LM, Woods SC, Benoit SC.** Gonadal hormones determine sensitivity to central leptin and insulin. *Diabetes*. 2006; 55:978–987.
24. **Nohara K, Zhang Y, Waraich RS, et al.** Early-life exposure to testosterone programs the hypothalamic melanocortin system. *Endocrinology*. 2011;152:1661–1669.
25. **Ryan K, Haller AM, Sorrell JE, Woods SC, Jandacek RJ, Seeley RJ.** Perinatal exposure to bisphenol-a and the development of metabolic syndrome in CD-1 mice. *Endocrinology*. 2010;151:2603–2612.
26. **Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM.** Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocr Rev*. 2009;30:75–95.
27. **Olofsson LE, Pierce AA, Xu AW.** Functional requirement of AgRP and NPY neurons in ovarian cycle-dependent regulation of food intake. *Proc Natl Acad Sci USA*. 2009;106:15932–15937.
28. **Lamont EW, Patterson Z, Rodrigues T, Vallejos O, Blum ID, Abizaid A.** Ghrelin-deficient mice have fewer orexin cells and reduced cFOS expression in the mesolimbic dopamine pathway under a restricted feeding paradigm. *Neuroscience*. 2012;218:12–19.
29. **Abizaid A, Liu ZW, Andrews ZB, et al.** Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. *J Clin Invest*. 2006;116:3229–3239.
30. **Schmittgen TD, Livak KJ.** Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3:1101–1108.
31. **Holson RR, Pearce B.** Principles and pitfalls in the analysis of prenatal treatment effects in multiparous species. *Neurotoxicol Teratol*. 1992;14:221–228.
32. **Tanaka M, Kawamoto T, Matsumoto H.** Distribution of 14C-bisphenol A in pregnant and newborn mice. *Dent Mater*. 2010;26:7.
33. **Doerge D, Vanlandingham M, Twaddle N, Delclos K.** Lactational transfer of bisphenol A in Sprague-Dawley rats. *Toxicol Lett*. 2010; 199:372–378.
34. **Shi H, Sorrell JE, Clegg DJ, Woods SC, Seeley RJ.** The roles of leptin receptors on POMC neurons in the regulation of sex-specific energy homeostasis. *Physiol Behav*. 2010;100:165–172.
35. **Riant E, Waget A, Cogo H, Arnal JF, Burcelin R, Gourdy P.** Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. *Endocrinology*. 2009;150:2109–2117.
36. **Pinto S, Roseberry AG, Liu H, et al.** Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science*. 2004;304:110–115.
37. **Kim MS, Pak YK, Jang PG, et al.** Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. *Nat Neurosci*. 2006;9:901–906.
38. **Monje L, Varayoud J, Luque EH, Ramos JG.** Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor α transcripts with alternative 5′-untranslated regions in the female rat preoptic area. *J Endocrinol*. 2007;194:201–212.
39. **Khurana S, Ranmal S, Ben-Jonathan N.** Exposure of newborn male and female rats to environmental estrogens: delayed and sustained hyperprolactinemia and alterations in estrogen receptor expression. *Endocrinology*. 2000;141:4512–4517.
40. **Williams KW, Margatho LO, Lee CE, et al.** Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. *J Neurosci*. 2010;30:2472–2479.
41. **Newbold RR, Padilla-Banks E, Snyder RJ, Phillips TM, Jefferson WN.** Developmental exposure to endocrine disruptors and the obesity epidemic. *Reprod Toxicol*. 2007;23:290–296.
42. **Hao CJ, Cheng XJ, Xia HF, Ma X.** The endocrine disruptor diethylstilbestrol induces adipocyte differentiation and promotes obesity in mice. *Toxicol Appl Pharmacol*. 2012;263:102–110.
43. **Yamamoto M, Shirai M, Tamura A, et al.** Effects of maternal exposure to a low dose of diethylstilbestrol on sexual dimorphic nucleus volume and male reproductive system in rat offspring. *J Toxicol Sci*. 2005;30:7–18.
44. **Dohler KD, Coquelin A, Davis F, Hines M, Shryne JE, Gorski RA.** Pre- and postnatal influence of testosterone propionate and diethylstilbestrol on differentiation of the sexually dimorphic nucleus of the preoptic area in male and female rats. *Brain Res*. 1984;302:291–295.
45. **McCarthy MM, Arnold AP.** Reframing sexual differentiation of the brain. *Nat Neurosci*. 2011;14:677–683.
46. **vom Saal FS, Welshons WV.** Large effects from small exposures. II. The importance of positive controls in low-dose research on bisphenol A. *Environ Res*. 2006;100:50–76.
47. **Ruhlen RL, Howdeshell KL, Mao J, et al.** Low phytoestrogen levels in feed increase fetal serum estradiol resulting in the "fetal estrogenization syndrome" and obesity in CD-1 mice. *Environ Health Perspect*. 2008;116:322–328.
48. **Cederroth CR, Vinciguerra M, Kuhne F, et al.** A phytoestrogen-rich diet increases energy expenditure and decreases adiposity in mice. *Environ Health Perspect*. 2007;115:1467–1473.
49. **Le HH, Belcher SM.** Rapid signaling actions of environmental estrogens in developing granule cell neurons are mediated by estrogen receptor ss. *Endocrinology*. 2010;151:5689–5699.
50. **Xi W, Lee CK, Yeung WS, et al.** Effect of perinatal and postnatal bisphenol A exposure to the regulatory circuits at the hypothalamus-pituitary-gonadal axis of CD-1 mice. *Reprod Toxicol*. 2011;31:409–417.
51. **Watanabe M, Ohno S, Nakajin S.** Effects of bisphenol A on the expression of cytochrome P450 aromatase (CYP19) in human fetal osteoblastic and granulosa cell-like cell lines. *Toxicol Lett*. 2012; 210:95–99.
52. **Ekman DR, Hartig PC, Cardon M, et al.** Metabolite profiling and a transcriptional activation assay provide direct evidence of androgen receptor antagonism by bisphenol a in fish. *Environ Sci Technol*. 2012;46:9673–9680.