Perinatal High-Fat Diet and Bisphenol A: Effects on Behavior and Gene Expression in the Medial Prefrontal Cortex

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Keywords

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Abstract

Both high-fat diets (HFD) and bisphenol A (BPA), an environmental endocrine disruptor, are prevalent in industrialized societies. Previous studies have detected separate effects of BPA and HFD; however, none have assessed possible interactive effects. Here, pregnant dams consumed 0, 40, or 400 µg BPA/kg/day and were fed either a control (CON; 15.8% kcal fat) or HFD (45% kcal fat) from gestational day 2 through parturition. The pups were individually dosed with BPA from postnatal days (P) 1–10, while the dams continued to consume one of the two diets. Maternal behavior increased with the HFD while the offspring's periadolescent social play decreased with BPA, but no interactive effects were observed. Neither HFD nor BPA exposure changed performance on a social recognition task, and only BPA had an effect on the elevated plus maze. BPA increased several cytokines in the medial prefrontal cortex (mPFC) of P10 males but not females. Expression of several genes related to hormone synthesis and receptors, inflammation, oxidative stress, and

apoptosis in the mPFC on P10 and P90 were altered due to BPA and/or HFD exposure with rare interactive effects. BPA resulted in an increase in the gene expression of *Esr1* in the mPFC of females on both P10 and P90. Epigenetic analysis on P90 did not show a change in methylation or in the levels of pre-mRNA or microRNA. Thus, perinatal BPA and HFD have separate effects but rarely interact.

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Introduction

The early environment, including maternal care, can have long-lasting effects on the cellular composition [1] and behavior [2] of rodent offspring with broad generalization to other mammalian species [3, 4]. Research in animal models has suggested that endocrine disruptors and dietary intake can alter maternal behavior, offspring behavior, and gene expression. Both endocrine disruptors and high-fat diets (HFD) have also been independently found to induce inflammation in the brain and periphery [5–7]; however, few studies have investigated their possible interactions [8]. This may have implications for humans given the ubiquitous exposure of endo-

crine-disrupting chemicals in our environment and HFD in Western society.

Endocrine disruptors are compounds that interact with hormone receptors as agonists, antagonists, or a mixture of both. Thus, endocrine disruptors can inappropriately activate a hormone receptor, even when the hormone is not present, and they can block the actions of hormones. Given that hormones play a role in the development of the nervous system and that hormonal receptors are present during this crucial time, endocrine disruptors can alter the course of development. One such endocrine disruptor is bisphenol A (BPA), a chemical found in polycarbonate plastics, certain dental sealants, and epoxy resins that line canned foods [9]. BPA binds with the highest affinity to estrogen-related receptor γ (ERR γ), but also to estrogen receptor (ER) α , ER β , and androgen and thyroid receptors [10–14]. Exposure to BPA has been found to change gene expression, neuroanatomy, and several types of behavior. Maternal behaviors during BPA exposure alter the time spent licking and grooming the pups as well as the time spent nursing in an arched-back position [15, 16]. Perinatal exposure to BPA also causes long-term effects on social, sexual, and anxiety behavior in adolescent and adult animals, as well as the number of neurons in a number of neural areas, including the medial prefrontal cortex (mPFC) [17–22]. At a molecular level, perinatal BPA exposure has been found to affect gene expression of *Esr1*, *Esr2*, *Errg*, as well as dopamine and serotonin receptor-related proteins in the PFC of young rats on postnatal day (P) 21 and mice on P28 [16, 23]. Furthermore, the differences in gene expression of *Esr1*, *Esr2*, and *Errg* have been found to be due to changes in DNA methylation in juvenile mice [16]. Based on the evidence of expression and methylation changes within the cortex and behavior changes in adolescence and adulthood, perinatal BPA exposure has a significant effect on the offspring.

HFD can also affect behavior, inflammation, and gene expression. The existing literature presents conflicting evidence of its effect on maternal behavior with both increases and decreases of positive maternal behaviors being reported [24–26]. In offspring, there are indications of HFD influencing social play and cognitive and anxiety behaviors [27–29]. Gestational HFD with 60% fat content has been found to increase proinflammatory cytokine expression of IL-1 β , microglial activation in the hippocampus of adult male and female offspring [28], and the level of IL-6 in the frontal cortex of adult male offspring [30]. HFD (45%) has also been found to alter gene expression in the hippocampus and performance on the Morris wa-

ter maze [31] as well as signaling in cultured primary neural stem cells [32]. Research on nonhuman primates also found alterations in the gene expression of serotonin receptors in male and female offspring following maternal HFD (32% fat) [29]. Gestational HFD (60% fat) through P40 is known to affect expression of oxytocin-related genes in the mPFC on P40 [27], and maternal HFD (60% fat) through gestation and lactation also alters opioid and dopamine-related proteins in the mPFC of adult male mice [33]. Few studies have investigated the effect of HFD and BPA on gene expression of hormone receptors in the PFC despite interactions of estrogen receptors and inflammation in other brain areas [34].

Although separate exposure to HFD and BPA is known to alter maternal behavior, offspring behavior, gene expression, and inflammation in the brain and periphery [5–7], the combined effects of both factors have not been investigated. Additionally, previous work in our laboratory found a higher number of neurons and glia in the mPFC of adult male, but not female, rats following perinatal BPA exposure [22]. The higher number of neurons found in male rats may have a parallel in the higher number of neurons found in a subset of children with autism, a male-biased neurological disorder with impaired social interactions as a core characteristic [35]. To further investigate this similarity, the current study focuses on the gene expression and cytokine levels in the mPFC, as well as social behaviors. We examine the possible interactive effects of BPA exposure and HFD during gestation and the early postnatal period on maternal care, as well as offspring behavior, gene expression, and cytokine levels in the mPFC.

Methods

Breeding

Male and female Long Evans hooded rats ($n = 58$ of each sex) purchased from Harlan Laboratories (now Envigo, Indianapolis, IN, USA) were housed in our vivarium for at least 1 week before being paired for breeding. They were kept on a 12:12 light/dark cycle and were allowed food and water ad libitum. Precautions were taken to reduce the environmental exposure to endocrine disruptors. All animals were housed in BPA-free polysulfone cages and fed either a low phytoestrogen diet (Harlan 2020X; Teklad Diets, Madison, WI, USA) or the specialized diet (see Diet) during gestation through P10; water was reverse osmosis filtered in glass bottles. All procedures were approved by the University of Illinois Institutional Care and Use Committee and adhere to the National Institute of Health guidelines on the ethical use of animals.

Breeding pairs were placed in suspended wire bottom cages and checked daily for the presence of sperm plugs. The breeding animals were paired for a maximum of 6 nights. If no sperm plug was

Fig. 1. Timeline of behavior and tissue collection following perinatal BPA and HFD exposure.

detected after 6 nights, the male was removed and another male introduced. The day a sperm plug was detected was recorded as gestational day (G) 0, and the dams were singly housed in a polysulfone shoebox cage. From the first day of pregnancy, each female was assigned to 1 of 6 groups. The number of litters for the control diet and BPA exposures were: 0 μg BPA/kg (0 BPA) = 8; 40 μg BPA/kg = 10; and 400 μg BPA/kg = 10. The number of litters for HFD and BPA exposures were: 0 BPA = 10; 40 μg BPA/kg = 10; and 400 μg BPA/kg = 10. The experimental design is illustrated in Figure 1.

BPA Dosing

BPA was suspended in tocopherol-stripped corn oil at 0, 0.1, or 1.0 mg BPA/mL in order to administer 0 (control), 40, or 400 μg BPA/kg, respectively. Our laboratory has previously found effects of the 40- and 400- μg BPA/kg doses [22, 36]. Tocopherol-stripped corn oil has no estrogenic activity as evidenced by no increase in uterine weight of prepubertal mice [37]. To dose the adult dams, the required amount (0.4 $\mu\text{L/g}$ body weight) was pipetted onto half a cookie (Newman's Own organic alphabet cookie, vanilla flavor) and given to the animals. The animals readily consumed the cookie making this route of exposure nonstressful and similar to human ingestion of BPA. On G0 and G1, the dams were given half a cookie with 0.4 $\mu\text{L/g}$ tocopherol-stripped corn oil. Starting on G2 through parturition, the dams were given the cookie with the assigned BPA dose. The day of birth was recorded as P0, and the litters were not disturbed. Then, daily from P1 to P10, each pup was individually dosed the assigned solution (same as dam) via pipetting directly into its mouth because lactational transfer of BPA is very low [38]. A plastic pipette tip was gently eased into the pup's mouth towards the back of the tongue. The oil was dripped onto the pup's tongue, and the experimenter slowly administered small amounts of oil to avoid damaging oral tissue and pulmonary aspiration. The pups easily and quickly consumed the small volume of oil (0.4 $\mu\text{L/g}$ body weight). Although the resulting level of BPA in the brain was not measured in the current study, previous research has shown that oral administration results in BPA being present in brain tissue of both fetal and early postnatal animals [39, 40].

Diet

Starting on G0 through P10, the dams were fed either a control diet (CON; D10012G, Research Diets Inc., NJ, USA) or HFD (D12451, Research Diets Inc.). The CON diet comprised 15.8% kcal fat, 20.3% kcal protein, and 63.9% kcal carbohydrate. The HFD consisted of 45% kcal fat, 20% kcal protein, and 35% kcal

carbohydrate. This diet was chosen because there is evidence that the Western diet has 35% fat on average [41], making the 60% HFD out of range for most people. The animals were allowed access to food ad libitum. Numbers and sex ratio of the pups and daily body weight for dams and pups were recorded.

Maternal Behavior Observations

The dams were observed with their litters from P3 to P10 during the dark cycle using night vision goggles by an observer blind to treatment groups. The dams' behavior was recorded every 3 min for 90 min (30 observations/night). The behavioral classifications were nursing, licking of pups, pup retrieval, nest building, and being away from the nest. When nursing and licking of pups occurred simultaneously, licking was preferentially recorded. Pup retrieval and nest building were rarely observed and, therefore, not included in the analyses.

Periadolescent Social Behavior

Two animals of the same sex and treatment (not cage mates) were paired to assess social behavior. The method of examining play in pairs of same-treatment animals is often used in the literature [42, 43]. Animals between ages P26 and P40 were isolated in individual cages for 1 h before each session and were then paired for social assessments in a neutral cage on 4 consecutive days 1 h before the start of the dark cycle. The animals' behavior was observed for 20 min, and 1-min time point sampling was used to classify the behaviors: sniffing, wrestling, chasing, passive contact (no play, but touching), and solitary (no play and not touching). After data collection, the animals were returned to their home cage. All animals in the study were assessed, and then the average from each sex within a litter was used in the statistical analysis.

Elevated Plus Maze

On P90 (adulthood), 1 male and 1 female from each litter were tested for anxiety behavior using the elevated plus maze (EPM). The floor of the EPM apparatus was 50 cm tall with 2 open arms and 2 closed arms that were each 50 cm long and 10.5 cm wide. The 2 closed arms had walls 33.5 cm tall. Each animal was placed on the center and allowed to explore the maze for one 5-min trial. Time spent in each arm, time in the center, and the number of entries into each arm were recorded. The operational definition of "entry" was more than half of the animal's body had to enter the arm in order to be counted and timed.

Social Recognition

On P90, another set of littermates not tested (see Elevated Plus Maze) were assessed for social memory in the social recognition task using same-sex juveniles (P21–P30). Before the task began, each juvenile was marked on its back with a differently colored nontoxic marker for easy identification. There were 3 days of habituation, which were identical to testing except the delay period was always 30 min. Data were collected during habituation but were not included in the data analysis. Following habituation, the delay periods (15, 45, 90, or 120 min) were randomized between animals for the 4 days of testing. To test social memory, adult animals were introduced and allowed to investigate a same-sex juvenile for 30 s. After the initial introduction, the juvenile animal was removed, and the adult was placed back with its cage mate for a delay period. After the delay period, the same juvenile (familiar) and a novel juvenile were placed in a cage with the adult. The time the adult spent investigating each juvenile was recorded for 3 min. In theory, an animal with good social memory will spend more time investigating the novel juvenile rather than the familiar juvenile. The adult animals never saw the same juveniles on more than 1 day.

ELISA

On P10, 1 male and 1 female from each litter were removed 1 h after BPA treatment. The remainder of the litter was allowed to age into adulthood. Each pup was anesthetized using CO₂ and then quickly decapitated. The brain was immediately removed and snap frozen in liquid nitrogen. The PFC was excised while the brain was frozen and 1 hemisphere was used for ELISA while the other was used for gene expression analysis (RNA Isolation and 1-Step Real-Time Quantitative PCR). The hemispheres were stored in a –80 °C freezer. All tissue was coded to conceal BPA treatment. A precoated ELISA plate (Signosis, Santa Clara, CA, USA) was used to analyze the levels of cytokines. Each plate could bind and measure tumor necrosis factor (TNF) α, transforming growth factor (TGF)-β, MCP-1, interleukin (IL)-1α, IL-1β, IL-6, IL-15, and vascular endothelial growth factor (VEGF) simultaneously, allowing for a survey of inflammatory factors. The second hemisphere of the P10 mPFC was lysed following the manufacturer's instructions. Briefly, 1 mL of cell lysis buffer/100 mg of tissue was homogenized on ice. The lysate was centrifuged to separate the tissue from the supernatant, which was then diluted to 100 µg/100 µL per well and used for the protein analysis. The samples were run in duplicate and incubated in the plate overnight followed by 3 washes. A biotin-labeled antibody (specific to each cytokine) was added and allowed to incubate for 4 h followed again by 3 washes. Next, a streptavidin-HRP conjugate was added to each well, incubated for 45 min, and followed by 3 washes. Finally, HRP substrate was added and allowed to incubate for 30 min before the addition of a stop solution to each well. The optical density of each well was determined with a microplate reader at 450 nm within 5 min.

RNA Isolation and 1-Step Real-Time Quantitative PCR

Genes related to hormone receptors (*Esr1*, *Esr2*, *Errg*, *Ar*), steroid synthesis (*StAr*, *Ldlr*, *Cyp11a1*), inflammation (*Aif1*, *C4a*, *C4b*), apoptosis (*Casp3*), and oxidative stress (*Cat*, *Sod1*, *Sod2*, *Glrx*) were chosen for investigation. Before genetic analysis, 1 hemisphere from each animal was randomly selected, and total RNA was isolated using TRI reagent (Sigma, St. Louis, MO, USA),

followed by Direct-zol™ RNA MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using the StepOne-Plus™ real-time PCR system with Power SYBR® Green PCR master mix (ThermoFisher Scientific, Waltham, MA, USA) using the respective forward and reverse primers for each gene (online suppl. Table S1; see www.karger.com/doi/10.1159/000494879 for all online suppl. material), and were designed by Vector NTI software (ThermoFisher Scientific) and synthesized by Integrated DNA Technologies (Coralville, IA, USA). Standard curves with a slope of –3.30 (SEM 0.30) and $R^2 \geq 0.99$ were accepted. A house-keeping gene ribosomal protein L7a (RpL7a) whose expression was not affected by treatment was used to normalize the gene expression data.

MRI, RNA Isolation, and 1-Step Real-Time Quantitative PCR

On P90, 1 male and 1 female from each litter were scanned for fat and lean mass in a magnetic resonance imaging (MRI) system. Following the scan, the animals were euthanized via CO₂, and the brain was immediately removed. The cortex was excised, snap frozen in liquid nitrogen, and stored at a –80 °C in a freezer. All tissue was coded to conceal the BPA treatment. The same genes analyzed in the P10 group were analyzed, and identical methods were used.

Genomic DNA Isolation and Methylation-Sensitive PCR Analysis of *Esr1*

Frozen cortex (50 mg) from P90 animals was ground in liquid nitrogen, and genomic DNA was then isolated using ZR Genomic DNA™ Tissue MiniPrep (Zymo Research, Irvine, CA, USA) per manufacturer's instruction. Genomic DNA was bisulfite converted using the EZ DNA Methylation-Gold™ kit (Zymo Research) following the manufacturer's instructions. Quantitative PCR (qPCR) used 20 ng of genomic DNA as the template using Power SYBR Green PCR Master mix as the reporter. Primers used for each genomic region were designed using the MethPrimer website [44], to create 4 primer sets that targeted the previously described promoter of *Rattus norvegicus Esr1* (ENST00000440973.1) [16]. Primer information is in the online supplementary Figure S1. A serial dilution from a reaction combining 1 sample from each experimental group was used to create an internal standard curve for quantification. Data are presented as the percent methylation

$$\text{Methylation (\%)} = 100/[1 + 2^{[1.33 - [\text{CtUnmeth} - \text{CtMeth}]/-2.27]}]$$

Identification of miRNAs Targeting *Esr1* 3'-UTR

Further exploration of epigenetic mechanisms was undertaken by analyzing the expression levels of 3 microRNAs (miRNAs) associated with *Esr1*. To identify rat miRNAs that might target *Esr1* 3'-UTR, a 3-pronged analysis was used. First, a bioinformatic approach was used to map the 3'-UTR of the *R. norvegicus Esr1* gene in miRDB (<http://www.mirdb.org/>), TargetScan (<http://www.targetscan.org/>), and PicTar (<http://pictar.mdc-berlin.de/>) to predict miRNAs that interact with *Esr1*. Then, a miRNA family that is broadly conserved across vertebrates targets the *Esr1* gene and bound by BPA was found, to facilitate potential generalizations across species in the future. The final miRNA candidates were selected based on families that overlapped in the 3 approaches: rno-mir-19b-3p (MIMAT0000788), rno-mir-221/222-3p (MIMAT0000890), and rno-mir-22-3p (MIMAT0000791). Quantifi-

Fig. 2. Maternal behavior assessment (data shown as means $\pm$ SEM in this and all subsequent figures). **a** Dams exposed to CON diet spent more time away from the nest compared to the HFD-exposed dams, $*p < 0.05$. There was no effect of BPA. **b** Dams exposed to CON diet spent marginally less time nursing the pups, $p = 0.055$. The data are collapsed across BPA treatment because there was no effect of BPA exposure. **c** Dams exposed to 400- μ g BPA/kg spent marginally less time licking the pups than 0-BPA dams, $p = 0.057$. The data are collapsed across diet because there was no effect of diet.

cation of specific miRNA was achieved through individual reverse transcription of previously isolated RNA for enrichment, followed by qPCR with the commercially available TaqMan probes for rno-mir-19b-3p (assay ID: rno478264_mir [cat. No. A25576]), rno-mir-221-3p (assay ID: rno481005_mir [cat. No. A25576]), and rno-mir-22-3p (assay ID: rno481004_mir [cat. No. A25576]), following the manufacturer's instructions. All miRNA content was normalized to a U6 housekeeping snRNA (NCBI accession: NR_004394; assay ID: 001973 [cat. No. 4440887]). Specific localization of each miRNA within the 3'-UTR of the *Esr1* rat gene (4037 bp) is illustrated in online supplementary Figure S2, where only rno-mir-19b-3p and rno-mir-22-3p showed at least 2 individual binding sites along this region. Seed sequences and binding sequences within *Esr1* are shown in online supplementary Figure S2.

Statistical Analyses

The growth curve for the dams' gestational body weights was analyzed with a 3 (BPA treatment) $\times$ 2 (diet) repeated-measure linear mixed model with a first-order ante-dependence covariance structure (determined by small Akaike's information criterion). Litter size, sex ratio, as well as each maternal behavior (nursing, licking, and time away from nest) were analyzed with 3 (BPA treatment) $\times$ 2 (diet) ANOVAs. For every measure, only 1 animal of each sex was used or the animals within the litter were averaged, and litter was the unit used for the analysis (e.g., social play). A 3 (BPA treatment) $\times$ 2 (diet) $\times$ 2 (sex) ANOVA was used to analyze the difference in pup weights on both P1 and P10. The analysis of MRI data was completed on each sex separately given the known sex difference in body weight.

To analyze the periadolescent social behavior, sniffing, chasing, and wrestling were combined into one "social play behavior" category. In order to use only 1 measure per litter, the average counts of each category per litter were calculated and used in the analysis. Each behavior was averaged across days 1–2 and 3–4. A 3 (BPA treatment) $\times$ 2 (diet) $\times$ 2 (sex) $\times$ 2 (days) repeated-measure ANOVA was used to analyze the play behavior data. Significant interactions were further analyzed using Tukey's LSD post hoc test.

A 3 (BPA treatment) $\times$ 2 (diet) $\times$ 2 (sex) ANOVA was used to analyze the EPM data. The same analysis was used to assess the percent of time spent investigating the novel juvenile at each delay of the social recognition task and the gene expression data col-

lected from the P10 and P90 cortex. To analyze the ELISAs, percent change from the control group (CON diet/0 BPA) was calculated for each cytokine, and then a 3 (BPA treatment) $\times$ 2 (diet) $\times$ 2 (sex) ANOVA with plate number as a cofactor was used to analyze the percent change for each cytokine. Tukey's LSD was used for post hoc tests. When analyzing the effects of BPA treatment, each dose was only compared to the control (0 BPA) group. Significant differential expression of miRNA data was defined as a fold change greater than 0.5.

Results

Maternal and Offspring Characteristics

Neither BPA nor HFD had a significant effect on gestational body weights, litter size, or sex ratio (data not shown). There were also no significant differences in offspring body weights on P1; however, BPA treatment differences emerged by P10, $F(2, 106) = 5.48$, $p = 0.005$, with the 40- μ g BPA/kg group weighing less than the 0-BPA group ($p = 0.002$). There were no effects on body weight or body composition in adult rats as assessed by MRI (data not shown).

Maternal Behavior

The HFD dams spent more time in the nest than the CON diet dams; $F(1, 52) = 0.25$, $p = 0.016$ (Fig. 2a). There was also an increase in the amount of time the HFD dams spent nursing the pups that was close to significant, $F(1, 52) = 3.84$, $p = 0.055$ (Fig. 2b). There was no effect of BPA treatment and no interaction on these measures. BPA had a near to significant effect on licking behavior, $F(2, 52) = 3.03$, $p = 0.057$ (Fig. 2c), with the 400- μ g BPA/kg exposure group spending less time licking the pups than the 0-BPA group ($p = 0.055$). Licking was not affected by diet and there was no interaction.

Fig. 3. BPA and social behavior separated into days 1–2 and 3–4 of testing sessions collapsed across sex because there were no interactions between sex and BPA. The data are also collapsed across diet. **a** 0-BPA animals engaged in more play behavior than the animals

exposed to 400 µg BPA/kg/day. **b** The animals exposed to 40 µg BPA/kg/day spent more time alone than the 0-BPA animals. **c** Animals exposed to 400 µg BPA/kg/day engaged in more passive contact than did the 0-BPA animals.

Periadolescent Social Behavior

There was a significant interaction between day and BPA treatment for play, $F(2, 160) = 6.34$; $p < 0.01$ (Fig. 3a). No significant differences were found on days 1 and 2 of play behavior; however, BPA treatment differences emerged on days 3 and 4. Play behaviors (sniffing, wrestling, and chasing) were significantly higher in the 0-BPA animals than in the 400-µg BPA/kg animals ($p < 0.05$), while the 40-µg BPA/kg animals also appeared to show a decrease in play; the difference with the control animals was not significant. There was also a main effect of sex in the amount of play behavior, with males exhibiting more play than females, $F(1, 160) = 6.70$, $p < 0.05$. There were no main effects of BPA treatment or diet.

There was a significant day-treatment interaction for time alone, $F(1, 160) = 4.00$, $p = 0.047$ (Fig. 3b). Again, days 1–2 were not different, while on days 3–4, the 40-µg BPA/kg animals spent more time alone than the 0-BPA animals ($p < 0.05$). There was a significant main effect of sex in the amount of time spent alone, $F(1, 160) = 14.08$, $p < 0.001$, with females spending more time alone than males. There were no significant differences in time alone based on maternal diet.

For passive contact, there was also a significant interaction between days and BPA treatment, $F(2, 160) = 5.67$, $p < 0.01$ (Fig. 3c). Once again, the differences did not occur on days 1–2 but on days 3–4; the 400-µg BPA/kg animals spent significantly more time in passive contact than the 0-BPA group ($p < 0.05$). There were no significant main effects of BPA or diet.

In each of the social behaviors, there was no significant BPA treatment-sex interaction, which would have suggested an attenuation or introduction of sex differences due to BPA treatment.

Elevated Plus Maze

There were no significant differences due to BPA or diet in the EPM for any of the measures (time in open arms, time in closed arms, number of crossings into the open arms, and number of crossings into the closed arms). There was a significant sex difference in the number of entries into open arms, with females entering the open arms more often, $F(1, 100) = 8.52$, $p < 0.01$ (Fig. 4). This is a frequently observed sex difference in the EPM [45].

Previous research has shown a reversal of sex differences with BPA-exposed males becoming less anxious [46]. In order to compare our results to previous literature, the analysis was restricted to the CON diet animals. There was a significant sex-BPA treatment interaction in the time spent in the closed arms, $F(2, 62) = 3.84$, $p < 0.05$, and time spent in the open arms, $F(2, 62) = 3.69$, $p < 0.05$. Post hoc analysis found a significant sex difference between males and females in the time spent in the closed arms ($p < 0.01$) and time spent in the open arms ($p < 0.01$), and 400 µg BPA/kg/day abolished the sex difference. Males showed a significant dose-dependent increase in the time in open arms with BPA treatment, $F(2, 35) = 3.70$, $p < 0.05$, suggesting an anxiolytic effect of BPA. Females did not show significant differences due to BPA treatment.

Fig. 4. Perinatal BPA (0, 40, or 400 µg/kg/day) and HFD exposure did not have a significant long-term effect on behavior in the EPM (see also the section Elevated Plus Maze for CON animals alone). **a** Time in the open arms. **b** Time in the closed arms.

Fig. 5. Perinatal BPA and HFD exposure did not result in significant long-term alterations in social recognition in adult animals (collapsed across sex). Means $\pm$ SEM.

Social Recognition

There were no significant differences in social recognition based on either BPA or diet ($p > 0.05$; Fig. 5).

Cytokine Levels in the P10 mPFC

The results of the ELISAs on the mPFC of P10 pups are shown in Table 1 as a percent change compared to control animals (CON diet/0 BPA). There were main effects of sex in 7 of the 8 cytokines examined. Males had a higher percent change than females in the level of TNF α , TGF β , MCP-1, IL-1 α , IL-15, and VEGF. Females had a higher percent change in the IL-6 cytokine level than males. The only cytokine that did not show a sex difference was IL-1 β . There was a significant main effect of diet on IL-6, $F(1,$

70) = 11.25, $p < 0.002$. There were no main effects of BPA on the levels of cytokines, but there were significant interactions between sex and BPA exposure (Fig. 6): TNF α , $F(2, 68) = 4.09$, $p < 0.03$; MCP-1, $F(2, 65) = 5.93$, $p < 0.005$, and VEGF, $F(2, 68) = 6.81$, $p < 0.003$. LSD post hoc comparisons found that males, but not females, had increased levels of TNF α , MCP-1, and VEGF following BPA exposure. Males had a significant increase in TNF α following both 40 and 400 µg BPA/kg ($p < 0.03$ and $p < 0.001$, respectively) and in MCP-1 and VEGF following 400 µg BPA/kg ($p < 0.006$ and $p < 0.003$, respectively).

In order to assess the possible associations between maternal behavior and cytokine levels of the offspring, a two-tailed Spearman's correlation was conducted com-

Table 1. Cytokine levels (percent change from control diet/0 BPA) in the mPFC of P10 pups with or without BPA treatment (0, 40, or 400 µg/kg/day)

Cytokines	Control diet		High-fat diet			ANOVA
	40	400	0	40	400	
TNFα						
M	13.4±5.9	10.7±9.8	-2.15±2.0	-4.2±5.7	12.0±8.1	Sex $p < 0.01$
F	5.0±6.7	7.3±5.9	3.8±3.8	-2.6±3.8	4.6±5.9	Sex × BPA $p = 0.02$
TGFβ						
M	1.6±5.2	8.0±9.7	9.3±8.4	-9.9±9.1	1.6±7.9	Sex $p = 0.05$
F	0.9±5.9	4.1±6.3	1.9±6.9	-4.5±4.2	2.2±8.7	
MCP-1						
M	4.6±5.8	3.3±5.5	0.4±3.6	-1.2±4.1	13.2±7.5	Sex $p < 0.001$
F	0.2±5.7	6.9±4.2	8.9±7.6	7.4±4.2	-0.3±3.9	Sex × BPA $p < 0.01$
IL-1α						
M	11.1±3.9	1.2±6.1	4.8±3.4	-3.9±3.4	14.1±5.3	Sex $p < 0.03$
F	6.3±4.3	2.1±5.3	-0.02±7.3	-0.9±3.3	0.5±4.2	
IL-1β						
M	8.7±8.9	11.8±8.7	-7.9±3.8	-2.5±3.3	13.9±10.0	
F	12.0±10.0	14.3±8.8	5.1±7.9	1.0±4.5	7.7±6.8	
IL-6						
M	12.4±7.5	20.8±9.1	0.6±7.4	-3.9±2.4	15.7±12.3	Sex $p < 0.001$
F	13.9±8.4	18.6±7.8	5.3±11.5	3.5±5.7	8.0±8.4	Diet $p < 0.001$
IL-15						
M	10.5±7.2	6.2±8.9	-1.9±3.4	1.2±8.3	15.7±9.4	Sex $p < 0.05$
F	18.0±5.5	5.8±6.1	-7.1±7.7	-0.8±4.1	3.5±4.3	
VEGF						
M	10.3±4.9	6.2±7.8	-0.6±2.2	-5.7±5.1	16.9±6.5	Sex $p < 0.001$
F	6.9±2.9	-2.3±7.9	-4.6±6.7	-4.7±5.0	-0.08±2.9	Sex × BPA $p < 0.01$

Values are expressed as the mean percent change from control (0 µg/kg/day BPA/control diet) ± SEM. M, males; F, females.

paring maternal licking to each protein. Maternal licking was negatively correlated with levels of IL-1α ($r = -0.257$, $p < 0.02$), IL-15 ($r = -0.243$, $p < 0.03$), and VEGF ($r = -0.249$, $p < 0.02$). The remaining cytokines were not significantly correlated with maternal licking.

Gene Expression in the P10 mPFC

The results of the qPCR analyses of the P10 mPFC are listed in Table 2. Females had higher expression than males in: *Esr1* ($p < 0.05$), *Sod1* ($p < 0.04$), *Aif1* ($p < 0.02$), *C4a* ($p < 0.001$), and *Casp3* ($p < 0.005$). There were main effects of BPA exposure in *Esr1*, $F(2, 89) = 7.39$, $p < 0.002$, and *Sod1*, $F(2, 89) = 3.75$, $p < 0.03$. Post hoc comparisons found that animals exposed to 40 µg BPA/kg ($p < 0.02$) and 400 µg BPA/kg ($p < 0.001$) had higher expression levels of *Esr1* in the PFC than 0-BPA animals (Fig. 7a); likewise, *Sod1* expression was significantly higher following both 40 and 400 µg BPA/kg than 0 BPA ($p < 0.02$). HFD exposure resulted in a significant decrease in gene expression of *Sod2* $F(1, 89) = 6.85$, $p < 0.02$.

The only significant interactions in gene expression between BPA treatment and diet were found in *Errg*, $F(2, 89) = 4.47$, $p < 0.02$ (Fig. 7b), and *Glrx*, $F(2, 89) = 3.46$, $p < 0.04$ (Fig. 7c). Post hoc analysis showed that the HFD/0-BPA group had higher *Errg* levels than both CON diet/0 BPA ($p < 0.001$), HFD/40 µg BPA/kg/day ($p < 0.05$). *Glrx* was higher following 400 µg BPA treatment only in the HFD group ($p < 0.05$). The remainder of the genes did not show significant differences (*Esr2*, *Ar*, *Star*, *Cyp11a1*, *Cat*, and *C4b*).

In order to assess the possible associations between maternal behavior and resulting gene expression of the offspring, a two-tailed Spearman's correlation was conducted comparing each maternal behavior to each gene. None of the behaviors or gene expression levels were significantly correlated (data not shown).

Gene Expression in the P90 Cortex

The results of the gene expression analysis of the cortex on P90 are shown in Table 3. Females had a higher

Fig. 6. The levels of proinflammatory cytokines that increased in males but not in females in the mPFC on P10 following perinatal BPA exposure (significant interactions between BPA and sex). The data are collapsed across diet. **a** Males exposed to 40 and 400 μg BPA/kg/day had higher levels of TNFα than the 0-BPA group.

b Males exposed to 400 μg BPA/kg/day had higher levels of MCP-1 than the 0-BPA groups. **c** Males exposed to 400 μg BPA/kg/day had higher levels of VEGF than the 0-BPA group. * $p < 0.05$; ** $p < 0.006$. Means $\pm$ SEM.

Fig. 7. Alterations in gene expression in the mPFC of P10 male and female pups following perinatal BPA (0, 40, or 400 μg/kg/day) and HFD. **a** *Esr1* expression increased with perinatal exposure to BPA (BPA: $p < 0.002$). The data are collapsed across diet. **b** *Errg* expression was higher in HFD/0-BPA animals than in CON diet/0-BPA

animals and in HFD/40-μg BPA/kg/day BPA animals (diet $\times$ BPA: $p < 0.02$). **c** *Glrx* expression was increased in animals exposed to HFD and 400 μg BPA/kg/day compared to HFD/0-BPA animals. No changes occurred in CON diet groups (diet $\times$ BPA: $p < 0.04$) * $p < 0.05$; ** $p < 0.01$.

level of gene expression in *Sod2* ($p < 0.001$), *Aif1* ($p < 0.04$), and *Casp3* ($p = 0.05$), while males had higher levels of gene expression in *C4a* ($p < 0.001$) and *C4b* ($p < 0.05$). HFD resulted in an increase in the expression of genes related to hormone receptors, oxidative stress, and apoptosis, including *Esr1*, $F(1, 66) = 4.63$, $p < 0.05$; *Errg*, $F(1, 66) = 4.37$, $p < 0.05$; *Cat*, $F(1, 66) = 8.92$, $p < 0.005$; and *Casp3*, $F(1, 65) = 4.74$, $p < 0.05$. There were no main effects of BPA on any of the genes analyzed.

However, BPA interacted with HFD in several genes: *Ar*, $F(2, 66) = 3.82$; $p < 0.05$; *Esr2*, $F(2, 66) = 3.40$; $p < 0.05$; *Glrx*, $F(2, 64) = 3.48$; $p < 0.05$. Post hoc tests on *Ar* showed a significant decrease in gene expression following perinatal HFD exposure compared to CON diet only in the 0-BPA group ($p < 0.02$). Further, *Ar* was significantly decreased between 0 and 40 μg BPA/kg/day ($p = 0.05$) only in the CON diet animals. Post hoc tests on *Esr2* showed that only within the CON diet groups was there a significant reduction in the level of gene expression at 400 μg compared to

Table 2. Gene expression from the mPFC of P10 male (M) and female rats (F) with or without BPA treatment (0, 40, or 400 µg/kg/day)

Gene expression	Control diet			High-fat diet			ANOVA	
	0	40	400	0	40	400		
Hormone receptors and enzymes								
<i>Esr1</i>								
M	0.74±0.06	0.99±0.02	0.97±0.06	0.85±0.04	1.01±0.06	1.08±0.04	Sex <i>p</i> < 0.05 BPA <i>p</i> < 0.002	
F	0.98±0.04	0.99±0.06	1.05±0.04	0.96±0.06	0.94±0.04	1.13±0.11		
<i>Esr2</i>								
M	0.88±0.09	0.93±0.10	0.98±0.11	1.20±0.10	0.80±0.10	0.79±0.08	Diet × BPA <i>p</i> < 0.02 Diet × BPA <i>p</i> < 0.02	
F	0.81±0.09	0.75±0.06	0.75±0.06	0.89±0.12	0.95±0.11	0.88±0.10		
<i>Errg</i>								
M	0.59±0.09	0.91±0.18	0.91±0.17	1.24±0.24	0.78±0.14	0.79±0.21		
F	0.52±0.06	0.64±0.08	0.75±0.10	1.08±0.15	0.87±0.11	0.82±0.16		
<i>Ar</i>								
M	1.02±0.10	0.95±0.07	0.89±0.09	0.84±0.09	0.88±0.09	0.95±0.10		
F	0.85±0.09	0.76±0.05	0.80±0.08	0.85±0.09	0.83±0.06	0.88±0.10		
<i>Star</i>								
M	1.32±0.13	1.06±0.09	1.13±0.07	1.21±0.09	1.20±0.08	1.15±0.08		
F	1.46±0.09	1.17±0.08	1.20±0.11	1.22±0.11	1.13±0.08	1.19±0.11		
<i>Cyp11a1</i>								
M	0.91±0.12	0.65±0.10	1.10±0.21	1.14±0.21	1.29±0.23	1.18±0.28		
F	0.79±0.26	1.37±0.20	1.24±0.25	1.24±0.15	1.30±0.22	0.89±0.03		
Inflammation or oxidative stress markers								
<i>Cat</i>								
M	0.96±0.02	1.03±0.02	0.98±0.02	0.96±0.04	0.97±0.04	0.94±0.01		
F	1.03±0.04	1.00±0.02	1.04±0.03	0.98±0.02	0.98±0.01	1.04±0.06		
<i>Sod1</i>								
M	0.86±0.06	0.99±0.03	0.96±0.02	0.91±0.03	1.01±0.04	1.03±0.03	Sex <i>p</i> < 0.04 BPA <i>p</i> < 0.03	
F	1.00±0.01	1.04±0.04	0.97±0.05	0.96±0.02	1.03±0.03	1.07±0.04		
<i>Sod2</i>								
M	1.15±0.01	1.18±0.09	1.05±0.05	1.02±0.04	1.08±0.08	1.04±0.08	Diet <i>p</i> < 0.02	
F	1.18±0.08	1.25±0.11	1.04±0.08	0.97±0.08	0.93±0.11	0.94±0.15		
<i>Glrx</i>								
M	1.06±0.03	1.01±0.03	1.00±0.01	0.95±0.05	1.02±0.04	1.05±0.07	Diet × BPA <i>p</i> < 0.04	
F	1.18±0.06	0.93±0.16	0.83±0.15	1.06±0.05	1.10±0.04	1.24±0.12		
<i>Aif1</i>								
M	1.12±0.13	1.08±0.08	1.12±0.06	1.09±0.10	1.24±0.06	1.13±0.08	Sex <i>p</i> < 0.02	
F	1.26±0.11	1.23±0.09	1.31±0.10	1.39±0.10	1.17±0.14	1.44±0.11		
Complement pathway								
<i>C4a</i>								
M	0.99±0.11	0.78±0.04	0.83±0.08	0.82±0.05	0.89±0.04	0.93±0.07	Sex <i>p</i> < 0.001	
F	1.00±0.06	0.93±0.05	1.13±0.14	1.18±0.11	0.97±0.05	1.29±0.09		
<i>C4b</i>								
M	1.34±0.14	0.79±0.08	0.94±0.15	1.17±0.13	1.13±0.11	1.11±0.26		
F	0.95±0.18	1.15±0.14	1.19±0.12	1.24±0.19	1.08±0.13	1.46±0.24		
Apoptosis								
<i>Casp3</i>								
M	0.94±0.03	0.99±0.03	0.88±0.03	0.90±0.04	0.89±0.07	0.99±0.05	Sex <i>p</i> < 0.005 Diet × BPA <i>p</i> = 0.056	
F	1.00±0.05	1.12±0.04	1.03±0.07	0.96±0.05	1.00±0.03	1.14±0.13		
Data expressed as mean ratio to RpL7a ± SEM.								

Table 3. Gene expression from the mPFC of adult male (M) and female rats (F) at P90 with or without BPA treatment (0, 40, or 400 µg/kg/day)

Gene expression	Control diet			High-fat diet			ANOVA
	0	40	400	0	40	400	
Hormone receptors and enzymes							
<i>Esr1</i>							
M	0.92±0.12	1.00±0.06	0.81±0.08	1.10±0.10	0.98±0.08	0.88±0.11	Diet $p < 0.05$
F	0.84±0.06	0.84±0.12	1.01±0.19	0.86±0.14	1.20±0.12	1.32±0.08	BPA $\times$ sex $p < 0.05$
<i>Esr2</i>							
M	1.10±0.09	1.08±0.15	0.76±0.11	1.03±0.10	0.91±0.16	0.99±0.09	Diet $\times$ BPA $p < 0.05$
F	1.05±0.08	0.88±0.10	0.78±0.07	0.88±0.15	1.01±0.08	1.15±0.08	
<i>Errg</i>							
M	1.19±0.14	1.37±0.20	1.05±0.15	1.36±0.09	1.32±0.11	1.45±0.08	Diet $p < 0.05$
F	1.48±0.15	1.05±0.12	1.23 $\pm$ 0.11	1.41±0.08	1.50±0.13	1.29±0.05	
<i>Ar</i>							
M	1.00±0.11	0.93±0.13	0.74±0.09	0.84±0.06	0.88±0.03	0.85±0.06	Diet $\times$ BPA $p < 0.03$
F	1.13±0.07	0.81±0.10	1.00±0.10	0.84±0.09	1.07±0.10	1.03±0.05	
<i>Star</i>							
M	0.91±0.15	0.95±0.20	0.69±0.14	0.90±0.14	0.89±0.07	0.98±0.10	
F	1.20±0.14	0.81±0.10	0.98±0.14	0.83±0.12	1.01±0.09	1.06±0.03	
<i>Cyp11a1</i>							
M	1.05±0.24	1.26±0.19	1.24±0.54	0.77±0.25	1.16±0.41	1.13±0.49	
F	1.27±0.35	1.37±0.40	1.80±0.44	1.85±0.70	1.17±0.27	1.67±0.38	
Inflammation or oxidative stress markers							
<i>Cat</i>							
M	1.03±0.08	1.00±0.07	0.87±0.07	1.19±0.06	1.05±0.03	1.18±0.06	Diet $p < 0.005$
F	1.07±0.04	0.98±0.12	0.98±0.12	1.16±0.10	1.13±0.06	1.12±0.04	
<i>Sod1</i>							
M	1.00±0.07	1.00±0.08	0.95±0.04	1.07±0.10	0.97±0.02	0.98±0.05	
F	1.07±0.05	0.91±0.10	1.05±0.08	1.00±0.09	1.10±0.09	1.11±0.04	
<i>Sod2</i>							
M	0.81±0.08	0.69±0.14	0.78±0.10	0.83±0.12	0.81±0.09	0.61±0.09	Sex $p < 0.001$
F	0.89±0.06	0.97±0.14	0.98±0.12	0.85±0.13	1.26±0.12	1.27±0.16	
<i>Glrx</i>							
M	1.08±0.12	1.00±0.11	0.90±0.10	0.96±0.08	0.88±0.07	0.87±0.07	Diet $\times$ BPA $p < 0.05$
F	1.16±0.06	0.86±0.11	0.88±0.08	0.92±0.08	1.20±0.08	1.30±0.13	Diet $\times$ sex $p < 0.05$
<i>Aif1</i>							
M	1.04±0.15	1.16±0.18	0.91±0.13	1.00±0.09	0.94±0.11	1.17±0.07	Sex $p < 0.05$
F	1.14±0.13	1.17±0.07	1.10±0.14	1.26±0.15	1.30±0.15	1.28±0.05	
Complement pathway							
<i>C4a</i>							
M	0.84±0.17	1.16±0.10	0.93±0.13	1.19±0.16	1.00±0.09	1.34±0.19	Diet $\times$ BPA $\times$ sex $p < 0.05$
F	0.85±0.04	0.66±0.04	0.64±0.06	0.77±0.08	0.91±0.08	0.76±0.04	Sex $p < 0.001$
<i>C4b</i>							
M	0.90±0.08	0.91±0.15	0.93±0.24	0.82±0.10	0.81±0.14	1.06±0.09	Sex $p < 0.05$
F	0.81±0.04	0.59±0.09	0.80±0.11	0.61±0.07	0.91±0.11	0.74±0.10	
Apoptosis							
<i>Casp3</i>							
M	0.80±0.06	0.81±0.04	0.69±0.06	0.85±0.05	0.74±0.04	0.80±0.06	Diet $p < 0.05$
F	0.87±0.05	0.76±0.08	0.74±0.07	0.83±0.04	0.97±0.05	0.97±0.07	Sex $p = 0.05$
Data expressed as mean ratio to RpL7a $\pm$ SEM.

Fig. 8. The increased levels of gene *Esr1* expression in P90 females exposed to 400 µg BPA/kg/day compared to controls; this did not occur in male animals. The data are collapsed across diet. * $p < 0.02$.

0 BPA ($p < 0.02$). For *Glrx*, there was only a trend in post hoc tests between 0 and 400 µg BPA within the CON diet ($p = 0.057$). In addition, there was a HFD-sex interaction in *Glrx*, $F(1, 64) = 4.84$; $p < 0.05$. Female animals in the HFD/400-µg BPA group had a significant increase in the level of gene expression compared to the 0-BPA group ($p < 0.04$), while males did not have significant change.

A BPA-sex interaction was found in the expression of *Esr1*, $F(2, 66) = 3.61$, $p < 0.05$, and a post hoc test revealed an increase in the level of gene expression in females following 400 µg BPA/kg/day ($p < 0.05$), but there were no differences in males (Fig. 8). There was a three-way interaction of HFD, BPA, and sex in *C4a*, $F(2, 65) = 3.38$, $p < 0.04$. However, post hoc tests revealed no significant differences when treated groups were compared to controls (0 BPA). The interaction was driven by the different responses of females and males to diet and BPA, but none were significantly different from their own control.

No HFD or BPA effects or interactions were observed in the gene expression of *Aif1*, *Star*, *Cyp11a21*, *Sod1*, *C4b*, *Sod2*.

Esr1 Gene Expression, premRNA Content and Promoter DNA Methylation

The consistent increase in *Esr1* expression in females due to BPA exposure between P10 and P90 (Fig. 7a, 8) was further investigated through analysis of the methylation of the *Esr1* gene (*Esr1*) in the P90 mPFC tissue. Comparisons were made to males that did not have a long-term increase. The hypothesis was that the control (0-BPA) females had higher levels of methylation than those that had been exposed to BPA (especially at 400 µg BPA/kg/day). Despite the changes in gene expression, premRNA

(the immature transcript before splicing to mRNA) content of *Esr1* in the PFC was not altered for males or female animals (online suppl. Fig. S3). Additionally, analysis of CG-rich sequences contained in the promoter of the rat gene revealed 4 CpG dense islands and upstream regions. These regions were selected for DNA methylation analysis through methylation-specific PCR. Regions 1 (+2043 to +2131 bp) and 4 (+4755 to +4847 bp) showed the highest variation for females and males, but no significant effect was detected (online suppl. Fig. S1).

miRNA Analysis of *Esr1* in the mPFC

Analysis of the 3 miRNAs associated with *Esr1* revealed that the miRNA levels were not significantly altered by BPA administration as none of the miRNA expression levels were increased by 0.5 or more (online suppl. Fig. S4 and S5).

Discussion

The effects of simultaneous perinatal exposure to both BPA and HFD were investigated. BPA influenced adolescent play behavior, the EPM, and in the mPFC, inflammatory markers and gene expression on P10 and P90. HFD affected maternal behavior and the expression of a subset of genes. Notably, perinatal BPA and HFD rarely interacted on the measures in this study; the exception being the expression of a small number of genes that did not change in the same direction across ages.

Maternal Behavior

There were separate effects of HFD and BPA exposure on maternal behavior. The dams fed HFD spent less time away from the nest and marginally more time nursing. These effects of HFD on maternal care are supported by previous work showing an increase in nursing and decrease in resting away from nest with HFD ingestion [8, 26, but see also 24]. Thus 45% HFD during the metabolically demanding times of pregnancy and postnatal nursing can facilitate maternal behavior. BPA exposure at the higher dose, on the other hand, resulted in a near significant decrease in the amount of licking behavior exhibited by the dam. This mirrors a pattern in preliminary data from our laboratory in which 400 µg BPA/kg/day resulted in less licking than 40 µg BPA/kg/day [47]. During the maternal observations, BPA was being administered directly to the pups but not to the dams. Thus, the decrease in licking by the dams may be due to BPA-induced changes in the pups, perhaps by alterations in behavior or olfactory cues. In-

creased maternal licking of pups has been linked to multiple behavioral and cellular alterations in the offspring, including a decrease in the behavioral stress response [1]. This is supported in our data by the negative correlation between maternal licking behavior and a reduction in inflammatory cytokines IL-1 α , IL-15, and VEGF.

Offspring Behaviors

Perinatal BPA exposure resulted in several significant long-term alterations in offspring behavior, while perinatal exposure to 45% HFD did not in the current study. Periadolescent play behavior was decreased in animals exposed to BPA in the latter half of the 4 days of observation. Animals in the 0-BPA group spent more time engaged in play behaviors than the 400- μ g BPA/kg animals, while animals exposed to 40 μ g BPA/kg spent more time alone than 0-BPA animals, and animals exposed to 400 μ g BPA/kg spent more time in passive contact than the 0-BPA animals. These results of periadolescent play behavior are consistent with previous studies that found a decrease in play following 40- μ g BPA/kg exposure [48]. Ferguson et al. [49] also showed a pattern of decrease in play behavior following prenatal BPA exposure that did not reach significance at the lower doses used (2.5 and 25 μ g). Although overall sex differences were found with males spending more time playing and females spending more time alone, there were no sex-specific changes due to BPA in these measures.

Along with the striatum and medial amygdala, play behavior is mediated by the mPFC, with inactivation of either the prelimbic or infralimbic subregions of the mPFC significantly reducing play [50, 51]. Because play behavior was assessed weeks after BPA exposure ended, these data suggest that perinatal BPA exposure may alter the long-term structure of several neural regions including the mPFC.

Evidence of an anxiolytic effect on the EPM due to BPA exposure in males was only uncovered when the CON diet group was analyzed separately, which supports previous literature that does not have a diet component [46, 52, but see also 53, 54]. Male animals in the 0-BPA group spent more time in the closed arms, but the males given 400- μ g BPA/kg/day increased their time on the open arms to female levels. We found that perinatal exposure to 400 μ g BPA/kg/day abolished the sex difference with an anxiolytic effect in males, which is in agreement with the literature [52].

Anxiety behavior is influenced by the mPFC through the cortical-amygdalar circuitry [55]. Both excitotoxic lesions and infusions of muscimol into the mPFC, which

inactivate GABA_A receptors, reduce anxious behavior in the EPM [56, 57]. Alterations in the anatomy of the mPFC by BPA could also affect anxiety behavior displayed in the EPM, and further research into the neuroanatomical effects of BPA on the mPFC is currently underway.

Inflammatory Cytokines in the mPFC

There was evidence in the present study that male, but not female, offspring had increased levels of proinflammatory cytokines TNF α , MCP-1, and VEGF in the mPFC at the highest dose of BPA. The sex-specific response to perinatal BPA administration may be due to the sex differences in the immune system that are found early in development [reviewed in 58]. The increased presence of cytokines in the early developing brain may indicate inflammation due to BPA exposure. Inflammation may also shape the behavioral outcomes of the offspring. Previous research has found increases in anxiety-like behavior and IL-6 and TNF α levels following perinatal BPA exposure [6]. Here, the highest dose of BPA produced the increased levels of inflammation in the early postnatal male animals, but the adult male animals showed an anxiolytic effect instead of the predicted anxiogenic behavior on the EPM following BPA administration.

Interestingly, perinatal exposure to HFD resulted in a decrease in the protein level of IL-6 in the mPFC, which is contrary to what was reported in the hippocampus and frontal cortex of young animals [28]. However, our study was focused on the mPFC, and the impact of perinatal HFD may be region dependent. Also, the necessary handling of pups for BPA administration during the first 10 postnatal days could have masked effects since early handling has a potent influence on later behavior [59], and its effects on inflammation are unknown. The current study adds to the evidence that BPA affects cortical inflammation during the early postnatal period and does not appear to interact with a 45% HFD during this exposure period.

Gene Expression in the mPFC on P10 and P90

Of the genes analyzed, only *Esr1* was comparably altered on P10, at the end of exposure, and on P90 in females, which may indicate long-term changes due to BPA. At P10, perinatal BPA exposure significantly increased the gene expression of *Esr1* in the mPFC in both sexes. The increase in *Esr1* expression seen here is similar but larger than that reported by Kundakovic et al. [16] who found a marginally significant U-shaped curvilinear effect on P28 in mice. Our study included direct BPA administration to the pups from P1 to P10 in addition to gestational exposure, which may explain the difference in strength of the results. The levels of *Esr1*

mRNA expression start to decrease in normative studies of P10 rats [60, 61], but in the current study, animals exposed to the high dose of BPA had increased *Esr1* expression. Early developmental alterations in the expression of *Esr1* could change the sensitivity of the animals to estrogens, particularly if the increase in *Esr1* persists, as we observed in the present study. This has also been postulated by Cao et al. [62], who found increases in *Esr1* gene expression in the hypothalamus due to prenatal BPA exposure. On P90, *Esr1* continued to be higher in females that had perinatal BPA exposure. Interestingly, *Esr1* was the only hormone-related gene in which BPA effects were the same on both P10 and adulthood. The increase in the expression of *Era* in the mPFC of females persisted well after the final administration of BPA, which could suggest that BPA increases the level of protein transcription.

The consistent increase in *Esr1* expression between P10 and P90 in females exposed to BPA was further explored. Several epigenetic mechanisms have been studied in regard to BPA in the brain, such as DNA methylation [16, 63, 64] and histone modifications [65]. In the current study, no clear pattern of methylation was obtained within regions of the gene that defined BPA exposure. In line with this result, the premRNA, or unprocessed transcript, was not significantly different between BPA treatments, concordant with the low impact of DNA methylation on the transcriptional control of the *Esr1* gene. DNA methylation mediates polymerase II binding and transcription factor accessibility to regulatory regions; however, the lack of active transcription could indicate that changes in mRNA expression are due to downstream stabilizing or destabilizing epigenetic regulators such as miRNA. However, of the 3 miRNAs identified, none were significantly increased in 0-BPA females, suggesting a different miRNA or mechanism is responsible for the long-term increase in *Esr1* expression.

Gene expression of another hormone-related receptor (*Errg*) was also influenced by BPA and HFD at each time point but not in the same direction. On P10, HFD increased the level of *Errg* expression, but in combination with BPA, the levels were not changed. In adulthood, there was no effect of BPA on *Errg*, but HFD resulted in decreased expression. ERR γ is an orphan nuclear receptor, which does not bind estrogen [66] but strongly binds BPA [67]. It is likely that ERR γ is, directly or indirectly, a part of the mechanism of action for BPA effects. When activated, ERR γ is able to modulate estrogen receptor-dependent signaling and even stimulate estrogen response element-mediated transcription without the presence of another ligand [66]. ERR γ has been only recently discovered, and the physiological impact of alterations in

its gene expression is unknown. Higher levels of adiposity can increase the level of circulating estrogens [68] and the number of ER binding sites in peripheral tissues [69]. However, this is the first reported evidence of an increase in *Errg* expression with HFD. The dearth of information on the function of ERR γ leaves the physiological effect of the changes in its expression unclear.

There were several genes that changed their expression in the mPFC due to sex and perinatal exposure to BPA and HFD on either P10 or P90. Expression levels of genes related to oxidative stress and inflammation were affected by BPA and HFD on P10 and P90 but rarely at both ages. The levels of *Glxr* expression showed an interaction between BPA and HFD with similar patterns on P10 and P90, however, which was significantly different from 0-BPA. In addition, other genes related to inflammation were also altered on P90. *Cat* and *Casp3* were increased following perinatal HFD, while *C4a* was altered by HFD and BPA in a sex-specific manner. Males had an increased expression of *C4a* following perinatal exposure to both BPA and HFD; however, females generally showed a decreased expression of *C4a*. Notably, neither HFD nor BPA caused an overall increase in the inflammatory markers, which suggests that not all of the inflammatory pathways are affected by BPA or HFD under these conditions.

Conclusion

The present study showed that perinatal exposure to BPA has effects on adolescent social behavior that tend to occur at the higher dose. Inflammatory markers and gene expression in the mPFC were also affected. BPA effects were often sex specific and more reliable at higher doses. Of particular interest is the lasting effect of BPA on the expression of *Esr1* in the mPFC. The impact of increased *Esr1* expression in the mPFC is currently unknown but may indicate an increased response to estrogens. With regard to maternal diet, HFD (45% fat) restricted to the perinatal period had limited effects on the variables assessed in the offspring but did acutely affect the dams' maternal care. The effects of BPA and HFD rarely interacted in the current paradigm.

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