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Mice Exposed to Bisphenol A Exhibit Depressive-like Behavior with Neurotransmitter and Neuroactive Steroid Dysfunction

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Abstract

Fetal exposure to endocrine disrupting chemicals (EDCs) has been associated with adverse neurobehavioral outcomes across the lifespan and can persist across multiple generations of offspring. However, the underlying mechanisms driving these changes are not well understood. We investigated the molecular perturbations associated with EDC-induced behavioral changes in first (F1) and second (F2) filial generations, using the model EDC bisphenol A (BPA). C57BL/6J dams were exposed to BPA from preconception until lactation through the diet at doses (10 µg/kg bw/d-lower dose or 10 mg/kg bw/d-upper dose) representative of human exposure levels. As adults, F1 male offspring exhibited increased depressive-like behavior, measured by the forced swim test, while females were unaffected. These behavioral changes were limited to the F1 generation and were not associated with altered maternal care. Transcriptome analysis by RNA-sequencing in F1

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control and upper dose BPA-exposed adult male hippocampus revealed neurotransmitter systems as major pathways disrupted by developmental BPA exposure. High performance liquid chromatography demonstrated a male-specific reduction in hippocampal serotonin. Administration of the selective serotonin reuptake inhibitor fluoxetine (20 mg/kg bw) rescued the depressive-like phenotype in males exposed to lower, but not upper, dose BPA, suggesting distinct mechanisms of action for each exposure dose. Finally, high resolution mass spectrometry revealed reduced circulating levels of the neuroactive steroid dehydroepiandrosterone in BPA-exposed males, suggesting another potential mechanism underlying the depressive-like phenotype. Thus, behavioral changes associated with early life BPA exposure may be mediated by sex-specific disruptions in the serotonergic system and/or sex steroid biogenesis in male offspring.

Keywords

Bisphenol A; behavior; hippocampus; RNA sequencing; serotonin; dehydroepiandrosterone

1. Introduction

Mental health disorders are a major contributor to the global burden of disease. Of note, the World Health Organization (WHO) selected depression as the theme for the 2017 World Health Day campaign, bringing worldwide attention to this major global health concern (World Health Organization (WHO) 2017). While genetic risk factors have been identified for mental health disorders (Lohoff 2010), increased evidence in rodent and human studies has also demonstrated a role for environmental influences on gene regulation and subsequent disease risk (Delgado-Morales 2017). Additionally, accumulating evidence has linked fetal development with behavioral and mental health outcomes in later life. The developing brain represents a particularly vulnerable window to environmental injury given the incomplete formation of the blood-brain barrier as well as blunted mechanisms for detoxifying exogenous chemicals (Choudhary et al. 2003; de Wildt et al. 1999; Grandjean and Landrigan 2014).

The developing mammalian brain relies on sex steroid hormones to mediate the composition of neural structures and connections that control a broad spectrum of cognitive and behavioral outcomes in adulthood (Palanza et al. 2008). This organizational window in rodents, which occurs just before and after birth, corresponds to the second and third trimesters of pregnancy in humans (McCarthy 2008). Additionally, a sexually dimorphic increase in estrogen in the developing male brain, through the aromatization of gonadal testosterone, establishes male-specific neural circuitries in late gestation (Palanza et al. 2008). Disruptions in the hormonal milieu during central nervous system (CNS) development, therefore, could impact brain function in a sex-specific manner. Given the well-established role for sex steroid hormones in proper brain development and function, endocrine disrupting chemicals (EDCs) have been proposed as potential neurotoxicants (Grandjean and Landrigan 2014; McCarthy 2008).

One EDC of great interest given its ubiquitous presence in the environment is bisphenol A (BPA), a chemical commonly used in the manufacturing of polycarbonate plastics and epoxy

resins (Calafat et al. 2008). Given its high production volume, urinary concentrations of BPA are detectable in virtually all sampled individuals in the United States (Calafat et al. 2008). As an EDC, BPA is best studied for its estrogenic activity (Yamasaki et al. 2000), although additional modes of BPA action including disrupted steroidogenesis have also been reported (Hong et al. 2016; Peretz and Flaws 2013). In humans, maternal exposure to BPA during gestation is associated with altered behaviors in children, including sociability, anxiety, and depression in some, but not all, studies (Mustieles et al. 2015). In rodent models, offspring gestationally and/or lactationally exposed to BPA have also been reported to exhibit comparable behavioral deficits (Palanza et al. 2016). Interestingly, the effects of BPA in both humans and rodents have occasionally been reported to be sex-specific, although the affected sex varies by study (Mustieles et al. 2015; Palanza et al. 2016). Unfortunately, within human epidemiological investigations as well as experiments in rodent models, the heterogeneity in study design limits comparative analyses. Nevertheless, the available literature suggests early life BPA exposure can negatively impact behavior. The mechanisms underlying these changes, however, remain unclear.

The hippocampus has long been associated with emotional regulation (Bannerman et al. 2014) and is susceptible to BPA. Morphologically, developmental exposure to BPA is associated with impaired synaptogenesis and spinogenesis, two estrogen-mediated processes, that can persist into adulthood (Kimura et al. 2016; Tiwari et al. 2015; Wang et al. 2014; Xu et al. 2014). Molecular assessments of hippocampal gene expression changes following developmental BPA exposure have, until recently, utilized candidate approaches (Chen et al. 2015; Kumar and Thakur 2017; Kundakovic et al. 2015; Wang et al. 2014; Xu et al. 2014). Given the pleiotropic effects associated with BPA exposure, unbiased approaches have gained favor in the identification of major pathways responsible for exposure-associated behavioral outcomes (Arambula et al. 2016).

We previously reported whole brain-specific expression changes of clinically relevant imprinted genes at midgestation in offspring exposed to BPA *in utero* (Susiarjo et al. 2013). The postnatal molecular and behavioral consequences of exposure in our model, however, remain to be determined. Moreover, alterations in behavior have been reported to persist across multiple generations of offspring directly exposed to BPA as well as generations that have received no direct exposure, known as multi- and transgenerational effects, respectively (Wolstenholme et al. 2012, 2013). Other than social deficits, however, multi- and transgenerational behavioral phenotypes associated with developmental BPA exposure have not been reported.

The aim of the present study was to assess multigenerational adult behavioral consequences associated with developmental BPA exposure and to identify underlying molecular dysregulation contributing to the behavioral changes. We identified male-specific disruptions in affective behavior that were associated with reduced levels of the hippocampal serotonin and systemic dehydroepiandrosterone, a neuroactive steroid in the sex steroidogenesis pathway. The depressive-like phenotype in F1 generational males did not persist into the F2 generation.

2. Materials and Methods

2.1. Animals and Treatments

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. The animals used in this study were treated humanely and with regard for alleviation of suffering. Mice were maintained on a 12:12-h light/dark cycle, with lights on at 07:00 (based on a 24-hour time system) and temperature maintained at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Drinking water was provided in polypropylene bottles. To verify the robustness of phenotypes, a total of five cohorts from two different animal facilities were examined over a two-year period. When possible, experiments were performed on animals and tissues from multiple cohorts of exposures and has been denoted in the figure legends. A comprehensive summary of assays performed on adult animals of each sex and generation can be found in Supplemental Table 1. All sections of this report comply with ARRIVE guidelines (Kilkenny et al. 2010); a completed checklist is included (Supplemental Checklist 1).

BPA (CAS 80-05-7; Sigma 99% purity)-supplemented feed was purchased from *Envigo* (Madison, WI). The control diet was a modified, low phytoestrogen, AIN 93G diet (TD 95092 with 7% corn oil substituted for 7% soybean oil to minimize exposure to other estrogen-like compounds that could confound BPA-related effects), and BPA diets included 50 μg BPA/kg diet (lower dose BPA; TD 110337) and 50 mg BPA/kg diet (upper dose BPA; TD 06156) supplemented into the control diet to approximate exposures of 10 μg and 10 mg BPA per kg bw/d (Dolinoy et al. 2007). The levels of exposure fall below established reference doses for BPA that are considered safe for humans: the tolerable daily intake (TDI; 50 $\mu\text{g}/\text{kg}$ bw/d), defined by the European Food Safety Authority (Bolt and Stewart 2011), and the lowest observed adverse effect level (LOAEL; 50 mg/kg bw/d), defined by the United States Environmental Protection Agency (Integrated Risk Information System (IRIS) 1988). Moreover, we previously assessed circulating levels of BPA in F0 maternal serum (Susiarjo et al. 2013) and found levels to be comparable to human exposures (Schönfelder et al. 2002; Vandenberg et al. 2007).

Six-week-old virgin C57BL/6J female mice (designated F0 generation, purchased from The Jackson Laboratory) were randomly assigned to control or BPA-containing diets beginning two weeks prior to mating. At least six F0 females per treatment group were exposed per cohort. Dietary exposure, a common route of human exposure, minimizes stress and provides a more chronic exposure throughout the duration of the experiment. Females were mated to unexposed C57BL/6J male mice during each cohort of exposure. Upon detection of a vaginal plug, females were separated from males. No females were mated for more than two weeks. Exposures continued throughout mating, gestation, and lactation. We previously reported no differences in maternal food consumption between control and BPA-exposed dams (Susiarjo et al. 2015). The start of this exposure window coincides with the acquisition of epigenetic marks in late oocyte maturation that may be susceptible to environmentally-induced disruption and subsequently influence offspring health (Smallwood et al. 2011). Postnatal exposure to BPA was included in our study because the differentiation and development of the rodent brain corresponding to the second and third trimesters of human

fetal brain development extend into the early postnatal period (Palanza et al. 2016; Williams et al. 2013).

All F1 generation offspring were weaned on postnatal day (PND) 21 and maintained on control diets throughout the remainder of the studies. No differences in litter size or sex ratio were observed, and no culling of litters was performed. To generate F2 offspring, a subset of six-week-old F1 females (one to two females per litter) were randomly selected to be mated to unexposed males. Animals used for adult behavior testing, aged 16-20 weeks, were randomly selected. No F1 or F2 offspring were singly housed. Following behavior testing, mice were sacrificed by cervical dislocation and hippocampus rapidly dissected, flash frozen, and stored at -80°C until further analysis.

2.2. Behavior testing

F1 adult behavior testing was conducted on male and female mice aged 16-20 weeks. F2 adult behavior testing was performed on male mice only. Tests were conducted in order of least to most distressing and offspring were allowed at least one day of rest between tests. Equipment was wiped with 70% ethanol and allowed to dry between tests. High definition digital recordings of all behavior procedures were collected for offline analysis. All behavior testing was completed by two trained observers blind to group designation, and analyses were completed by one trained observer to ensure consistency across cohorts of behavior tests. We controlled for time-of-day effects (Roedel et al. 2006) by beginning and ending assessments at the same time across testing days. Adult behavior testing was initially conducted in males, and behaviors altered by early life BPA exposure were then assessed in females to address sex-specificity. For early-life behavior testing (i.e. mother-offspring behavior), cages were checked for parturition daily at 08:00. The day of birth was considered postnatal day one (PND1).

2.2.1. Hole Poke (HP)—Exploration of a 14×14 inch arena with a lattice of 16 holes in the floor was conducted in the light between 07:00 and 10:00 on adult F1 male and female offspring as well as F2 adult males. Exploratory and general locomotor activity was assessed by infrared light beam emitters and detectors within the holes and in a scaffold around the arena. A computer interface system (PAS-Open Field, San Diego Instruments) recorded hole pokes, horizontal and vertical beam breaks in a ten-minute trial.

2.2.2. Elevated Zero Maze (EZM)—The EZM was used to assess anxiety-related behavior in F1 adult male mice. This test was conducted in the light between 07:00 and 10:00. Mice were placed on an annular track elevated ~ 80 cm from the floor and divided into quadrants: 2 opposing quadrants were bordered on either side by 10cm high walls (considered the closed arms of the track) while the other 2 quadrants remained open. Individual mice were placed in a closed arm of the maze and recorded for a five-minute trial. Increased time spent in the open arms of the maze is interpreted as a reduction in anxiety-like behavior (Braun et al. 2011). Image analysis was performed on open source MatLab-based software (Patel et al. 2014). Time in the open and closed segments of the maze as well as the latency to enter an open arm during the trial were determined.

2.2.3. Social Choice (SC)—The SC procedure is used to identify impaired social interactions in adult F1 male mice. SC was conducted later in the day, between 10:00 and 14:00, and in low light (~10 lux), to enhance activity and exploration towards the inanimate and social cues during the assessment (Sankoorikal et al. 2006). A three chamber social choice arena was used as described elsewhere (Sankoorikal et al. 2006). Briefly, the arena is configured to contain ventilated plastic cylinders on either side of the arena. The cylinders are used to hold a non-social object (rock) and a social object (same sex gonadectomized A/J mouse) in opposite sides of the arena. After a ten-minute habitation to the arena with empty cylinders, a non-social and social cue were loaded in their respective cylinders for a ten-minute social preference phase. In a third phase of the procedure, the cylinders are removed and the mice are allowed to directly interact with the social cue mouse and the non-social cue for five minutes. The interaction phase time is graded for aggression. An open source program developed by David Meany (<http://www.seas.upenn.edu/~molneuro/autotyping.html>) was used to analyze the social choice procedure recordings: this analysis compares the amount of time that the tested mouse interacts with the target mouse during the second phase of testing.

2.2.4. Forced Swim Test (FST)—Porsolt's FST was performed in the light between 07:00 and 10:00. Adult male (F1 and F2 generations) and female (F1 only) mice were tested in 46 × 22 cm diameter glass cylinders filled to a depth of about 20 cm with water (25 ± 1°C) from which there was no escape. A single six-minute trial was performed. The first two minutes of the test were ungraded time to allow for acclimatization to the cylinder. During the remaining four minutes of the trial, total time spent immobile was recorded. Increased time spent immobile is sensitive to antidepressants and is related to depressive-type behavior (Cryan and Mombereau 2004; Porsolt et al. 1977). Following behavior testing, estrus stage (metestrus/diestrus, proestrus, estrus), was determined in F1 females by vaginal smears (McLean et al. 2012).

2.2.5. Rescue Experiment—The selective serotonin reuptake inhibitor fluoxetine hydrochloride (F132 Sigma) was resuspended in 0.9% saline at a concentration of 2 mg/mL. Beginning at 07:30, adult male mice (16 weeks) were administered saline or fluoxetine (20 mg/kg bw) intraperitoneally thirty minutes prior to FST. The dose of fluoxetine (20 mg/kg bw) was selected based on previous studies using C57BL/6 mice of comparable ages (Bahi et al. 2014; Tang et al. 2014).

2.2.6. Nest quality—Maternal care was assessed in F0 dams on the day of parturition. Assessments of nest quality were conducted at 08:00 on PND1. One compressed cotton square was provided to each dam for nest building. The scoring system used has been described elsewhere (Hess et al. 2008). In brief, nests are scored based on the shape and height of walls built up around the pups. Scores range from 0 (undisturbed nesting material) to 5 (complete dome).

2.2.7. Pup retrieval—Pup retrieval was conducted in the light between 08:30 and 10:30 on PND1. Two pups were gently removed from the nest and placed in each of the corners furthest from the nest. Recordings lasted for 10 minutes. Latency to carry the first pup,

retrieve the first pup to the nest, and total time to return both pups to the nest were assessed as previously described (Myers et al. 1989). Additionally, the relative amount of time the F0 dam spent in the nest, irrespective of the type of behavior exhibited in the nest, and the time spent actively adjusting nest materials were noted (Myers et al. 1989; Palanza et al. 2002).

2.3. Tissue harvest and RNA isolation

Following behavior testing, mice were allowed to recover for at least one week prior to euthanasia. Adult mice were euthanized by cervical dislocation. Animals were sacrificed in random order. Whole blood was collected immediately after euthanasia and allowed to clot for 30 minutes at room temperature. Samples were subsequently centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant, designated serum, was quickly removed and stored at -80°C until further analysis. Whole hippocampal tissue from each hemisphere was individually dissected and flash frozen in liquid nitrogen to allow for multiple downstream molecular assessments per animal. To ensure consistency in whole hippocampal tissue isolations, the same researcher dissected each mouse.

Total RNA was extracted from whole hippocampus using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. RNA concentrations were determined using the Nano Drop spectrophotometer, and 350 ng of RNA was run on an agarose gel to assess RNA quality.

2.4. RNA-sequencing (RNA-seq)

Four control and four upper dose BPA adult (20 week) male hippocampal samples from behavior-tested mice were used for the RNA-Seq study. 5µg of total RNA was used as input for poly(A)+ selection using Dynabeads mRNA DIRECT purification kit (Ambion). Barcoded libraries were made using ScriptSeq v2 RNA-Seq Library Preparation Kit (Illumina) and ScriptSeq Index PCR Primers (Illumina). Library quality was assessed on an Agilent 2100 Bioanalyzer system and quantified using the Kapa quantification kit (Kapa Biosystems). Sequencing was performed on a NextSeq 500 platform (Illumina).

RNA-seq reads were trimmed for quality using Trimmomatic (version 0.32) (Bolger et al. 2014). Leading and trailing low quality (below quality 3) and N base calls were trimmed, and reads were scanned using a 4-bp sliding window and trimmed when the average quality per base dropped below 15. Reads with at least 30 bp in length following this quality control process were retained and used for subsequent analyses. The resulting reads were then aligned to mm10 reference genome assembly using STAR (version 2.3.0e) (Dobin et al. 2013), allowing read pairs to align no farther than 2000bp apart and keeping only unique, concordantly aligned read pairs for genome-wide transcript counting. FeatureCounts (version 1.5.0) (Liao et al. 2014) was used to generate a matrix of mapped fragments per RefSeq annotated gene. Numbers for total sequenced, aligned and counted reads for each sample are listed in Supplemental Table 2. Analysis for differential gene expression was performed using DESeq2 (Love et al. 2014) with the cutoff of FDR < 0.05 (Supplemental Dataset 1). Using these differentially expressed genes (DEGs) as input, we performed Core Analysis provided by the Ingenuity Pathway Analysis (IPA) software. This analysis interpreted our dataset in the form of canonical pathways and upstream regulators that were

overrepresented in the DEG list. All default parameters were used. Clustering and heatmap of differentially expressed genes were generated using heatmaply R package. The raw sequencing data reported in this work have been deposited in the NCBI Gene Expression Omnibus under accession number GSE102849.

2.5. Quantitative real-time PCR (qRT-PCR)

RNA was treated with RQ1 RNase-Free DNase (Promega) prior to cDNA conversion using Superscript III reverse transcriptase (Invitrogen) and random hexamers (Roche). qRT-PCR was run on a 7900HT Fast Real Time PCR system (Applied Biosystems). Samples were run in triplicate using the following recipe: 5 μ l of Power SYBR green PCR Master Mix (Applied Biosystems), 0.2 μ l of forward primer (10 μ M), 0.2 μ l of reverse primer (10 μ M), and 4.6 μ l of cDNA (5 ng). Whole hippocampal gene expression from behavior-tested and non-tested animals was normalized to *Tbp* and *Hprt*, and expression relative to control was calculated using the comparative Ct method. Primer sequences for the genes of interest can be found in Supplemental Table 3.

2.6. High performance liquid chromatography (HPLC) analysis of biogenic amines and amino acids

The F1 male and female adult hippocampal tissues from behavior-tested and non-tested animals were processed and analyzed by a researcher blinded to treatment. The hippocampus was sonicated for 10 seconds in homogenization buffer (22 mM sodium acetate, 74.8 mM glacial acetic acid, 0.05mM EDTA pH 4.95) containing 250nM 3,4-dihydroxybenzylamine (internal standard for biogenic amines) and 1mM L-alpha-amino adipate (internal standard for amino acids) then centrifuged at 14,000 rpm at 0°C for 30 min. An aliquot of the supernatant (30 μ l) was diluted with 5 μ l of 0.3mg/ml ascorbate oxidase and 20 μ l of the subsequent mixture was used for biogenic amine analysis as described previously (Stein et al. 2006). A second aliquot (10 μ l) was diluted 1:1 with 0.8 N perchloric acid (10 μ l) and analyzed for amino acids as described previously (Kilpatrick et al. 2010). In three samples, DOPAC was not well resolved, in three samples NE was not well resolved, and in one sample DA was not resolved; these values were excluded from the analysis prior to unblinding.

2.7. Liquid chromatography-high resolution mass spectrometry (LC-HRMS)

Testosterone, dehydroepiandrosterone (DHEA), and estrone assays were performed on 50 μ L serum from behavior-tested and non-tested animals after digestion with β -glucuronidase/arylsulfatase from *Helix pomatia* as previously described using LC-HRMS with $^{13}\text{C}_3$ -testosterone as an internal standard (Frey et al. 2016). The limit of quantification (LOQ) was 20 pg/mL serum for testosterone and DHEA and 25 pg/mL for estrone. In tissue, the LOQ for testosterone, DHEA, and estrone were 20 pg, 15 pg, and 5 pg per gram of tissue, respectively. Imputation of data values below the LOQ were performed as previously described (Hornung and Reed 1990; Succop et al. 2004).

2.8. Statistical analysis

Given the intralitter variability commonly seen in our exposure model (Susiarjo et al. 2013), we randomly selected at least two animals/sex/litter, when possible, for physiological and molecular studies. The adult offspring was the experimental unit. All values are reported as mean $\pm$ SEM. To account for the presence of multiple animals per litter, we used linear mixed effects modeling (Stata 14.2). P-values were adjusted for multiple comparisons. $p < 0.05$ was considered statistically significant.

Because the sex steroid data did not meet the assumption of normality, a non-parametric test was used. A Kruskal-Wallis H test was used to determine if sex steroid levels were different across the three treatment groups followed by the Dunn-Bonferroni post hoc method. Again, $p < 0.05$ was considered statistically significant.

Effect size estimates were calculated using Cohen's d for pair-wise comparisons and eta squared (η^2) for Kruskal-Wallis. Cohen's d was calculated using an effect size calculator found at <http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php> (Wellman et al. 2018). The η^2 estimate was calculated manually as previously described (Tomczak and Tomczak 2014).

3. Results

3.1. Early life exposure to BPA induces depressive-like behavior specific to F1 generation adult males

To determine the long-term behavioral consequences of developmental BPA exposure, we performed a battery of behavior tests assaying a broad range of cognitive functions, starting in F1 adult male mice. Previous reports from our lab have described male-specific effects of BPA exposure on metabolic outcomes (Bansal et al. 2017; Susiarjo et al. 2015). Outcomes affected by developmental BPA exposure in males were also assessed in females to determine sex-specificity.

Elevated Zero Maze (EZM) and Forced Swim Test (FST) assess anxiety and depressive-like behavior, respectively. There was no significant difference in the amount of time spent in the open versus closed arms in EZM or the latency to enter the open arm (Supplemental Fig. 1A-1C), suggesting levels of anxiety in BPA-exposed animals were comparable to controls in adulthood. However, FST revealed a significant increase in the time spent immobile in F1 males exposed to both lower (Fig. 1A; $p=0.01$, $d=1.12$) and upper dose BPA (Fig. 1A; $p < 0.01$, $d=1.25$), suggestive of a depressive-like state. The enhanced immobility was not due to hypoactivity or general locomotor impairment in lower and upper dose BPA males as assessed by Hole Poke (HP) (Fig. 1B). When age-matched F1 adult females were assessed for depressive-like behavior and general activity, no significant differences were observed across treatment groups (Fig. 1C and 1D). Stage of estrous did not affect the results of FST or HP in F1 females (data not shown). Developmental BPA exposure in our model, therefore, is associated with depressive-like behavior in males only.

In addition to affective behavior, sociability has been commonly assessed following developmental exposure to BPA (Dessi-Fulgheri et al. 2002; Jašarević et al. 2011; Porrini et

al. 2005; Wolstenholme et al. 2011a). In our exposure model, when given the choice to interact with a social or non-social object (mouse or rock, respectively), F1 adult male mice spent comparable proportions of time interacting with the social object, regardless of treatment group (Supplemental Fig. 1D). Thus, early life exposure to BPA in our model did not affect social behavior in F1 adult males.

Finally, the transmission of phenotypes across multiple generations has received considerable attention in the developmental origins of health and disease (DOHaD) and environmental exposure fields (Schmidt 2013). A limited number of multi- and transgenerational rodent studies examining behavioral endpoints following developmental BPA exposure have been reported, with a focus on altered sociability (Wolstenholme et al. 2012, 2013). To determine whether BPA exposure-associated affective behavior is transmitted to subsequent generations, we assessed FST and HP in F2 adult male offspring. F2 offspring were generated by breeding F1 exposed females to unexposed males. F2 adult males from BPA-exposed lineages had no significant differences in FST or HP relative to control males (Fig. 1E and 1F), limiting the behavioral effects of developmental BPA exposure in our study to the F1 generation.

3.2. Behavioral changes in F1 males following BPA exposure are independent of altered maternal care

Disruptions to the maternal hormonal milieu by synthetic chemicals can compromise maternal care and influence offspring behavior (Palanza et al. 2008). To assess the effect of BPA on maternal care, we analyzed both pup-directed and non-pup-directed behaviors of control- and BPA-fed dams on the day of parturition. We first assessed nest quality using a previously described scoring system that grades the nest based on its shape and height (Hess et al. 2008). Exposure to BPA did not affect nest quality (Supplemental Fig. 2A). To determine whether BPA exposure influenced pup-directed behaviors, we performed a pup retrieval test (Kuroda and Tsuneoka 2013; Myers et al. 1989). Two pups were separated from the cage and placed on the opposite end relative to the nest. We then assessed the time for the F0 dam to carry the first pup, return the first pup to the nest, and return all pups to the nest. Maternal exposure to dietary BPA did not affect any of the pup retrieval endpoints (Supplemental Fig. 2B-2D). Finally, we observed no significant differences in the amount of time the F0 mother spent in the nest or the time spent actively nesting (Supplemental Fig. 2E and 2F). Together, these results suggest that behavioral changes in BPA-exposed offspring are not a secondary consequence of differences in maternal care.

3.3. Transcriptome analysis in F1 adult male hippocampus suggests early life exposure to BPA alters neurotransmitter systems

To investigate potential mechanisms responsible for the BPA exposure-associated behavioral outcomes, we used RNA-sequencing (RNA-seq) to analyze the whole hippocampal transcriptome of control and upper dose BPA F1 adult male mice (n=4 per treatment group) in an unbiased manner. The hippocampus is a critical brain region involved in the regulation of mood (Bannerman et al. 2014). Approximately 92% of the trimmed reads aligned to the reference sequences and the number of mapped reads ranged from $39\text{--}76 \times 10^6$ per sample (Supplemental Table 2). The Principal Component Analysis (PCA) plot generated by the

expression profile of all genes did not show a separation between control and upper dose BPA samples (Supplemental Fig. 3A), indicating the expression differences between the two groups are small compared to individual differences. Despite the fact that gene expression fold changes were small (median fold change of all DEGs was 1.59), we identified 110 differentially expressed genes (DEGs) of statistical significance using a cutoff of $FDR < 0.05$ (Supplemental Fig. 3B, Supplemental Dataset 1), and unsupervised clustering of the DEGs stratified animals by their expected treatment groups (Fig. 2A). Ingenuity Pathway Analysis (IPA) revealed catecholamine biosynthesis and glutamate receptor signaling as top affected canonical pathways (Fig. 2B and 2C). The former pathway included an upregulated gene, tyrosine hydroxylase (*Th*), which is a rate-limiting enzyme in catecholamine biosynthesis. The latter pathway included a downregulated gene, ionotropic kainate glutamate receptor 5 (*Grik5*), which encodes a glutamate receptor subunit. The directionality of gene expression changes following developmental BPA exposure for *Th* and *Grik5* were confirmed by qRT-PCR (Fig. 2D).

3.4. Whole hippocampal serotonin levels are sex-specifically altered in F1 BPA-exposed males

The monoamine deficiency hypothesis postulates that reduced levels of serotonin (5-HT), norepinephrine (NE), and/or dopamine (DA) in the CNS are responsible for depression (Copen 1967; Schildkraut 1965). In addition to monoamines, agents targeting amino acid neurotransmitter systems such as glutamate have received increased attention as potential therapeutic strategies for treating depression (Rakesh et al. 2017). Based on RNA-seq results suggesting dysregulated catecholamine synthesis and amino acid neurotransmitter signaling following developmental exposure to BPA, we next asked whether these gene expression changes were sufficient to disrupt neurotransmitter levels. Using high-performance liquid chromatography (HPLC), we observed a dose-dependent decrease in 5-HT ($p=0.04$, $d=-0.89$) and the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA, $p=0.038$, $d=-0.821$) in upper dose BPA-exposed adult male whole hippocampus while females were unaffected (Fig. 3A and 3B). The ratio of 5-HIAA to 5-HT, a measure of 5-HT degradation, was not significantly affected in BPA-exposed males or females (Fig. 3A and 3B).

Levels of the catecholamine neurotransmitters NE and DA were sex-specifically altered by BPA exposure. DA was increased in lower dose BPA-exposed males (Supplemental Fig. 4A; $p=0.03$, $d=0.32$), and NE was reduced in lower dose BPA-exposed females (Supplemental Fig. 4C; $p=0.04$, $d=-0.52$). The dopamine metabolite homovanillic acid (HVA) was also reduced in lower dose BPA-exposed females (Supplemental Fig. 4C; $p=0.04$, $d=-0.59$). Finally, levels of amino acid transmitters aspartate (Asp), glutamate (Glu), gamma-aminobutyric acid (GABA), and glutamine (Gln) were not significantly different between the experimental groups in F1 males (Supplemental Fig. 4B and 4D). However, in F1 females, GABA was reduced in lower dose hippocampus (Supplemental Fig. 4D; $p=0.02$, $d=-0.99$). In sum, multiple neurotransmitters are dysregulated in a sex- and dose-specific manner following BPA exposure. Specifically, in BPA-exposed males, serotonin and catecholamine neurotransmitters appear to be more susceptible to disruption by early life exposure to BPA than amino acid neurotransmitters.

3.5. Fluoxetine rescues lower but not upper dose BPA exposure-associated behavior in F1 generation males

Antidepressant drugs are a mainstay of treatment in depression, with selective serotonin reuptake inhibitors (SSRIs) representing the most commonly prescribed class of antidepressants in humans (Cipriani et al. 2012; Nautiyal and Hen 2017). Fluoxetine (FLX), also known by the trade name Prozac, was among the first SSRIs used to treat depression (Nautiyal and Hen 2017). Fluoxetine allows for the targeted accumulation of serotonin in the synapse and provides increased opportunities for the transmitter to activate its downstream signaling pathways (Nautiyal and Hen 2017). Given the male-specific behavioral phenotype and reduction in hippocampal serotonin, we next asked whether the behavioral consequences of early life exposure to BPA could be rescued with FLX. Saline or 20 mg/kg bw of FLX was administered intraperitoneally to F1 adult males 30 minutes prior to FST. While lower dose BPA-exposed males responded to FLX administration (Fig. 3C; $p < 0.01$, $d = -1.32$), upper dose males did not exhibit significant improvements in FST (Fig. 3C), suggestive of treatment resistance. These results also suggest dose-specific molecular changes underlying the depressive-like phenotype in BPA-exposed F1 male offspring.

3.6. Circulating neuroactive steroids are depleted in BPA-exposed F1 adult male mice

In addition to neurotransmitter system dysfunction, hormonal imbalances are a well-established biological factor in the etiology of depression (Xu et al. 2015). Importantly, various intermediate hormones in the sex steroidogenesis pathway also function as neuroactive steroids and have potential roles in mood regulation (Maninger et al. 2009). Using LC-HRMS, we assayed circulating DHEA, testosterone, and estrone in F1 adult male serum to determine whether differences in systemic neuroactive steroids could be contributing to the depressive-like phenotype. Estrone was below the limit of quantification for all samples (data not shown), while testosterone was not significantly different across treatment groups (Fig. 4A). A Kruskal-Wallis H test revealed a significant difference in serum DHEA ($H(2 \text{ d.f.}) = 6.50$, $p = 0.039$, $\eta^2 = 0.12$). A Dunn-Bonferroni post hoc method subsequently identified a significant reduction in serum DHEA in lower dose BPA-exposed males (Fig. 4B; $p = 0.015$). Whole hippocampal tissues showed similar trends of reduced testosterone and DHEA, albeit not statistically significant (Fig. 4C and 4D). No gross differences in testis weight, the primary location for DHEA and testosterone synthesis in rodents (Maninger et al. 2009), were observed across treatment groups (Fig. 4C). The disruption of neuroactive steroids following developmental BPA exposure may, therefore, also be contributing to the observed depressive-like state in BPA-exposed F1 adult males.

4. Discussion

In this study, we investigated the underlying molecular changes associated with altered behavior following chronic, *in utero* and lactational exposure to the endocrine disruptor BPA. The proper action of sex steroids during a restricted developmental window, just before and just after birth in rodents, is required for the effective induction of behaviors in adulthood (McCarthy 2008). F1 adult male offspring developmentally exposed to BPA were significantly more immobile in the FST compared to sex-matched controls, suggestive of a depressive-like state. Importantly, these effects were not due to differences in maternal care,

although other exposure models have reported effects of BPA on parental care (Kundakovic et al. 2013). In this study, maternal care assessments are limited to the day of parturition. Future studies should extend to behaviors such as licking and grooming as well as specific types of nursing behavior (*e.g.* arched back, flat posture), and all observations should be repeated across multiple days. The observed depressive-like phenotype also occurred independent of an anxiety phenotype, which has been reported in some (Wolstenholme et al. 2011a), but not all (Rebuli et al. 2015; Williams et al. 2013; Wolstenholme et al. 2011b), studies. While BPA has been reported to induce transgenerational alterations in social behavior (Wolstenholme et al. 2012, 2013), affective behavioral changes in our study were limited to the F1 generation. The previously mentioned inconsistencies and others, such as the direction and sex-specificity of behavioral changes, associated with developmental BPA exposure may be attributed to heterogeneity in study design. This includes factors such as rodent strain, dose of BPA, route and window of exposure, age of offspring at time of assessment, as well as how the behavior tests are conducted (*e.g.* phase of light/dark cycle). Regardless, it is clear that the period of neurodevelopment represents a vulnerable window to environmental perturbations that have lasting effects on behavior.

Given the depressive-like phenotype in F1 adult males, we performed RNA-seq on the adult male whole hippocampus, an established brain region involved in mood regulation (Bannerman et al. 2014). This study is the first to report transcriptome-wide changes in the adult murine hippocampus following early life exposure to BPA. In another RNA-seq study using a different developmental BPA exposure model, only 10 genes were found to be differentially expressed in the male newborn rat hippocampus compared to 1000+ genes in the hypothalamus (Arambula et al. 2016), suggesting differential susceptibility of brain regions to BPA. Therefore, studies in other brain regions with roles in mood regulation, such as the amygdala, may provide additional mechanistic insight into our BPA exposure-associated behavioral changes. Of the 10 DEGs reported in the neonatal rat hippocampus by Arambula and colleagues (Arambula et al. 2016), none overlapped with our dataset, which is unsurprising given the differences in species and assayed developmental stage. Differences aside, the greater number and distinct set of DEGs in our adult hippocampal data set suggest additional changes in gene expression that become apparent following the induction of circulating sex steroid hormones. The directionality of gene expression changes from our RNA-seq study was validated by qRT-PCR; given the variability in response to developmental BPA exposure, this may have contributed to the lack of statistically significant differences in gene expression in our qRT-PCR studies (Fig. 2).

Pathway analysis of the DEGs identified neurotransmitter systems as top dysregulated pathways in the BPA-exposed adult whole hippocampus. Given the autoregulatory nature of neurotransmission (Nautiyal and Hen 2017), we hypothesized neurotransmitter levels themselves may be affected in BPA-exposed male mice. Hippocampal neurotransmitter analysis by HPLC identified sex- and dose-specific differences in serotonin, dopamine, norepinephrine, and GABA in the adult male and female hippocampus (Fig. 3, Supplemental Fig. 4). Altered serotonin levels were of particular interest given the sex-specificity in this study as well as previous work from our lab reporting effects of BPA exposure on tryptophan metabolism, the precursor of 5-HT (Susiarjo et al. 2017). One other report has assessed neurotransmitter levels in mice perinatally exposed to BPA (250 ng/kg/d), from gestation

day 10 to postnatal day 20 (Matsuda et al. 2013). While adult female mice demonstrated a significant decrease in hippocampal 5-HT turnover in their study, male hippocampal 5-HT levels were not affected (Matsuda et al. 2013). No assessments of affective behavior, however, were performed. In our study, both lower dose and upper dose BPA males exhibited increased depressive-like behavior and decreases in 5-HT, although reductions in 5-HT may be occurring through distinct mechanisms at each dose. It is tempting to speculate that the slight reduction in 5-HT in lower dose males in our study may be due to increased turnover, as shown by a moderately increased ratio of 5-HIAA to 5-HT (Fig. 3A). In contrast, decreased levels of 5-HT and 5-HIAA in the upper dose animals (Fig. 3A) suggests a general depletion of 5-HT in the hippocampus. Consistent with previous reports published by our lab, this result suggests that functional outcomes following developmental BPA exposure are driven by distinct, dose-specific molecular changes (Bansal et al. 2017; Susiarjo et al. 2017). Future assessments in brain regions that synthesize the respective neurotransmitters (e.g. dorsal raphe nucleus for 5-HT) will also help to more precisely identify the mechanisms of BPA exposure-associated neurotransmitter disruption.

Given the prominent role of serotonergic dysfunction in the etiology of depression (Nautiyal and Hen 2017), we asked whether the depressive-like state in BPA-exposed F1 males could be rescued by an anti-depressant targeting the serotonergic system. Interestingly, administration of 20 mg/kg bw FLX was sufficient to rescue the depressive-like phenotype in lower dose male mice while upper dose mice did not display significant improvement in the FST (Fig. 3C), again demonstrating dose-specific effects. In females, changes in neurotransmitter levels in the absence of a depressive-like phenotype could be due to insufficient changes in magnitude to elicit a detectable change in physiology. Alternatively, the changes in female-specific neurotransmitters could be associated with other behavioral outcomes that were not assessed in this study.

To determine other potential factors associated with the depressive-like phenotype in F1 adult males developmentally exposed to BPA, we asked whether levels of steroids in the sex steroidogenesis pathway were affected by developmental BPA exposure. Hormonal imbalance has been associated with affective disorders in both humans and rodents. Hypogonadal men have been reported to be at increased risk for depression, and castration in rodent models increases depressive-like behavior (McHenry et al. 2014). Previous studies in gonadal tissues have also reported suppression of critical enzymes in the steroidogenesis pathway following early life exposure to BPA (Hong et al. 2016; Peretz and Flaws 2013). In our study, BPA exposure was associated with a significant reduction in systemic DHEA levels in lower dose males while testosterone was not significantly different from controls at either dose (Fig. 4A and 4B). A similar trend in reduced steroid levels was observed directly in hippocampal tissue (Fig. 4C and 4D). Although steroid levels in serum and tissue in our study were variable, the average values were comparable to previous reports in unexposed animals (Hojo et al. 2011; Nilsson et al. 2015). Average serum testosterone levels in our study were slightly lower than Nilsson et al., at 5546 pg/mL in control males compared to 8235 pg/mL. On the other hand, average serum DHEA values in our study was higher (1842 pg/mL in control males) than DHEA levels reported by Nilsson and colleagues (<50 pg/mL). Discrepancies across studies are likely due to differences in methodology (Nilsson et al. 2015).

Because the hippocampus has been reported to be capable of synthesizing its own neuroactive steroids (Maninger et al. 2009; Tuem and Atey 2017), it is possible that the brain recognizes and compensates, somewhat, for the reduction in steroids from peripheral sources. Although not significantly different, the depleted hippocampal steroids in combination with the depleted neurotransmitters may be exacerbating the behavioral change following developmental exposure to BPA. While estrogen and testosterone are well-established neuroactive steroids and often the primary steroids of interest in EDC studies, upstream steroids like DHEA have been reported to exert distinct neurobiological activities. In addition to its neuroprotective and neurogenic effects, DHEA can modulate neurotransmitter function (Maninger et al. 2009). DHEA has been reported to be involved in catecholamine synthesis and secretion and glutamate receptor signaling, two pathways identified in our hippocampal RNA-seq data, as well as serotonin release (Lazaridis et al. 2011; Maninger et al. 2009; Tuem and Atey 2017). Although the genes encoding sex steroidogenesis enzymes did not show significant expression differences by RNA-seq, it is possible that their abundance and/or activity at the translational level could be affected by early life BPA exposure. In humans, low circulating levels of DHEA have been associated with increased ratings of depressed mood in some, but not all, studies (Maninger et al. 2009). Exogenous administration of DHEA, however, has consistently been reported to exert antidepressant effects in humans (Maninger et al. 2009), suggesting supplementation of DHEA aids in regulating mood via additional mechanisms beyond just correcting for deficiencies in systemic DHEA. Whether exogenous steroid supplementation in our animal model would rescue the depressive-like phenotype, and how the effects would compare to antidepressant therapy, remains to be determined.

Given its ability to mimic estrogen (Xin et al. 2015; Yamasaki et al. 2000), the involvement of classical and non-classical estrogen receptors (ERs) in the sex-specific depletion of hippocampal 5-HT following developmental BPA exposure also remains to be determined. It is known that estrogen can alter levels of 5-HT as well as regulate the number and function of its receptors (Kajta et al. 2017; Ryan and Ancelin 2012), suggesting BPA could be acting through ERs to mediate its effects. Although BPA can bind to the classical ERs, ER α and ER β , its affinity is lower than that of endogenous estrogen (Acconcia et al. 2015), suggesting classical ER-independent mechanisms of BPA action may also contribute to the observed phenotypes. Non-classical ERs such as the membrane-bound G protein-coupled estrogen receptor 1 (GPER1) have also been reported to modulate serotonergic function (Kajta et al. 2017) and BPA is capable of activating GPER1-mediated downstream pathways *in vitro*. Additionally, BPA has been reported to bind to GPER1 with greater affinity than either ER α or ER β (Fitzgerald et al. 2015; Xu et al. 2017). Interestingly, GPER1 knockout mice exhibit impaired affect and stress control, with more robust effects in male than female mice (Kastenberger and Schwarzer 2014). Although the transcripts for the classical and non-classical ERs were not shown to be significantly affected by BPA in our hippocampal RNA-seq dataset, it is possible that their regulation is also being affected at the translational level.

Finally, it will be worthwhile to determine if behavioral changes are detectable earlier than adulthood in our model. Transient developmental disruptions of serotonergic function using conditional knockout strategies and pharmacological inhibition have been shown to exert long-term consequences on adult affective behavior (Ansorge 2004; Gross et al. 2002;

Heisler et al. 1998; Toth 2003). Some reports suggest that the affective behavioral impairments following these disruptions impact males more than females (Heisler et al. 1998), although others report no apparent sex differences (Toth 2003). Assessing affective behavioral outcomes in animals prior to puberty will also provide insight into the requirement for circulating sex steroids in the detection of sex-specific affective behavioral changes.

5. Conclusion

In this study, we show that early life exposure to BPA, at levels representative of human exposure, elicits persistent, sex-specific behavioral consequences in adulthood. F1 male offspring of dams exposed to BPA from preconception until weaning demonstrate increased depressive-like behavior. This behavioral change is not due to altered maternal care and is not transmitted through the maternal germline to the F2 generation. Male-specific depletions in hippocampal serotonin and systemic neuroactive steroids may be responsible for the observed behavioral changes in the F1 generation. Understanding the effects of EDCs in early mammalian development and their mechanism(s) of action on a local and genome-wide scale will allow for improved assessments of their health risk to the general population and may aid in future regulatory decision-making. Overall, this study highlights the susceptibility of neurodevelopment to environmental exposures and its potential role in the genesis of mental health disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Developmental BPA exposure is associated with adult behavioral despair in F1 males
- Affective behavior in BPA-exposed F1 female and F2 males is not affected
- BPA exposure is associated with altered hippocampal neurotransmitter systems
- Antidepressant rescues BPA exposure phenotype in a dose-specific manner
- Neuroactive steroid, dehydroepiandrosterone, is reduced in BPA-exposed male serum

Figure 1.

Developmental exposure to BPA induces sex- and generation-specific affective behavior in adulthood. (A) Forced Swim Test (FST) and (B) Hole Poke (HP) in F1 males (circles). (C) FST and (D) HP in F1 adult females (triangles) demonstrate no significant differences across treatment groups. (E) FST and (F) HP in F2 adult males (diamonds). Behavior changes in F1 generation males did not persist to the F2 generation. Number of offspring and number of litters denoted below each treatment group from three cohorts of exposures. The adult animal was used as the unit of measurement. * $p < 0.05$; ** $p < 0.01$ compared to sex-

matched controls. Bars represent mean $\pm$ SEM. Linear mixed effects modeling with correction for multiple comparisons.

Figure 2. RNA-sequencing reveals disrupted hippocampal neurotransmitter systems following developmental BPA exposure. (A) Hierarchically clustered heat map of 110 differentially expressed genes (DEGs) from RNA-sequencing of control (abbreviation: C) and upper dose (abbreviation: U) BPA-treated F1 adult male hippocampus. N = 4 males per treatment group (from independent litters) from one exposure cohort. The expression level of 98 upregulated and 12 downregulated genes shown as Log2 of sequence read counts per million (CPM). A complete list of all genes can be found in Supplemental Dataset 1. Top canonical pathways by Ingenuity Pathway Analysis for (B) upregulated and (C) downregulated DEGs and affected genes. (D) qRT-PCR validation studies for tyrosine hydroxylase (*Th*) and ionotropic kainate glutamate receptor 5 (*Grik5*) in F1 adult male hippocampus. Fold changes by RNA-seq for *Th* and *Grik5* in upper dose BPA-exposed males were 1.4 and 0.69, respectively. By qRT-PCR, fold changes in upper dose were 1.24 and 0.74, respectively. Number of offspring and number of litters denoted below each treatment group from two cohorts of exposures. The adult animal was used as the unit of measurement. Bars represent mean $\pm$ SEM. Linear mixed effects modeling with correction for multiple comparisons.

Figure 3.

Developmental BPA exposure reduces hippocampal serotonin in males and depressive-like behavior is rescued by antidepressant targeting the serotonergic system. F1 adult (A) male (circles) and (B) female (triangles) hippocampal serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and the ratio as a measure of 5-HT turnover. In (A) and (B), legends below graphs correspond to number of adult offspring and litters (in parentheses) analyzed for each neurotransmitter and metabolite. Neurotransmitter analyses conducted on animals from three cohorts. (C) Saline (SAL, circles) or fluoxetine (FLX, squares) was administered in F1 adult males 30 minutes prior to FST. Number of offspring and number of litters denoted below each treatment group, from one exposure cohort. In all panels, the adult animal was used as the unit of measurement. * $p < 0.05$; compared to sex-matched controls. Bars represent mean $\pm$ SEM. Linear mixed effects modeling with correction for multiple comparisons.

Figure 4.

Circulating neuroactive steroids are depleted in BPA-exposed male mice. Serum (A) testosterone and (B) dehydroepiandrosterone (DHEA) measured by high resolution mass spectrometry from five exposure cohorts. DHEA was significantly reduced in upper dose BPA male serum. Hippocampal (C) testosterone and (D) DHEA from one exposure cohort show similar trends to serum. (E) Testis weights, from one exposure cohort, were not significantly different across treatment groups. Number of offspring and number of litters denoted below each treatment group. The adult offspring was used as the unit of

measurement. For (A-D), Kruskal-Wallis H test followed by Dunn-Bonferroni post-hoc test. For (E), linear mixed effects model with correction for multiple comparisons. * $p < 0.05$ compared to sex-matched controls. Lines denote mean $\pm$ SEM.