Rosenfeld Laboratory - Material and Methods

Description of the test articles used, study material evaluations (diet, drinking water, cage and bedding leachates), general study design, animal treatments, and animal allocations to the 2 year toxicology study conducted at NCTR and the grantee studies can be found in Heindel et al. (2015).

The methods below were published as part of our manuscripts: Johnson et al. (2016) and Cheong et al. (2018).

1. **BEHAVIOR TESTING:** Johnson SA, Javurek AB, Painter MS, Ellersieck MR, Welsh TH Jr, Camacho L, Lewis SM, Vanlandingham MM, Ferguson SA, Rosenfeld CS. Effects of developmental exposure to bisphenol A on spatial navigational learning and memory in rats: A CLARITY-BPA study. Horm Behav. 2016;80:139-148.

Animal Husbandry and Dosing

Comprehensive details on animal husbandry, treatment, and dosing procedures have been published (Heindel et al., 2015). Therefore, only brief details are described here. All animal use and procedures were approved in advance by the NCTR Institutional Animal Care and Use Committee and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited facility. Experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (Council, 2011). The animal rooms for breeding, gestation, and pre-weaning housing were maintained at 23 ± 3°C with a humidity level of 50 ± 20%, and a 12 hour:12 hour light/dark cycle with lights on at 6:00. After weaning on postnatal day (PND) 21, the light cycle was changed to a reverse cycle (light on at 11:00; off at 23:00). A low phytoestrogen diet (5K96 verified casein diet 10 IF, round pellets, γ-irradiated, Test Diets, Purina Mills, Richmond, IN), and Millipore-filtered water in glass water bottles with silicone stoppers (#7721 clear, The Plasticoid Co., Elkton, MD) were provided ad libitum. Housing cages were polysulfone with microisolator tops (Ancare Corp., Bellmore, NY) and contained hardwood chip bedding. Drinking water, cage, and bedding extracts were tested for BPA and none had levels detectable above the level of the average analytical blanks. Diet was tested for BPA, genistein, daidzein, zearalenone, and coumesterol, and only lots with < 5 ppb BPA, < 1 ppm genistein and daidzein, and < 0.5 ppm zearalenone and coumesterol were used. Breeder male and female Sprague-Dawley (SD) rats (i.e., F0) from the NCTR breeding colony were placed in the above conditions (e.g., low phytoestrogen diet, glass water bottles, polysulfone cages) at weaning on PND 21. SD rats

were chosen here for several reasons. They are one of the most common rodent strains in toxicological research, and the studies described herein are part of the larger CLARITY-BPA studies where all investigators are using this animal model (Birnbaum et al., 2012; Heindel et al., 2015; Schug et al., 2013). Additionally, SD rats are widely used in various Barnes maze experiments (Barrett et al., 2009b; Locklear and Kritzer, 2014; Morel et al., 2015), including those performed at the NCTR (Ferguson et al., 2012), and we sought to compare the current findings to those prior studies.

Another study suggested that adult SD perform better than Dark Agouti rats in this behavioral test (Barrett et al., 2009b). Finally, SD rats are easy to handle and are more resistant to injuries that might result from the gavage method that was used to dose the animals (described below) (Germann and Ockert, 1994; Germann et al., 1998; Germann et al., 1995).

Approximately 2 weeks prior to mating, females were assigned to one of five treatment groups (vehicle control, 2.5 BPA, 25 BPA, 2500 BPA, or EE) based on body weight ranking to produce approximately equal mean body weights in each group. Males were randomly mated with females with the stipulation that no sibling or first cousin pairing was permitted. Breeding occurred in five "loads" or "cohorts" each spaced four weeks apart. Offspring from the last two breedings (i.e., loads 4 and 5) were used for the behavioral studies described here and in Rebuli et al. (Rebuli et al., 2015).

Dams were considered pregnant when a sperm plug or a sperm-positive vaginal cytology was observed [mating day = gestational day (GD 0)]. Beginning on GD 6, dams were gavaged daily with 0.3% carboxymethylcellulose (CMC or vehicle), 2.5 µg BPA/kg bw/day, 25 µg BPA/kg bw/day, 2500 µg BPA/kg bw/day, or 0.5 µg EE/kg bw/day. These BPA doses were selected to provide low, middle, and upper levels of exposure and are below the no-observed-adverse-effect level (NOAEL) of 5 mg/kg bw/day as detailed in (Tyl et al., 2008; Tyl et al., 2002). These doses are also within the dose range used in to prior studies conducted at the NCTR with the same animal model (Delclos et al., 2014; Ferguson et al., 2014). The highest EE dose available in the CLARITY-BPA study was selected to better compare to a 10-fold greater dose (5 µg EE/kg bw/day) employed in a prior study (Ferguson et al., 2012). The materials used for dose formulation were BPA (CAS # 80-05-7, TCI America, Portland, OR; catalogue # B0494, Lot # 111909/AOHOK [air-milled], ≥ 99.9 purity), EE (CAS # 57-63-6, Sigma-Aldrich, St. Louis, MO; catalogue # E4876, Lot # 071M1492V, > 99% purity), and CMC (Sigma-Aldrich; catalogue # C5013, Lot # 041M0105V). Dams were gavaged daily at a volume of 5 ml/kg bw using a modified Hamilton Microlab® ML511C programmable 115V pump (Hamilton Co., Reno, NV; Lewis et al., 2010). No treatment occurred on the day of parturition, typically GD 22. On the day after birth, (i.e., PND 1), litters were randomly

culled to 5/sex where possible and identified with a distinguishable paw tattoo. Only offspring from litters born between GD 20–26 and containing at least 3/sex, a minimum of 9 live pups, and a sex difference not greater than 5 at birth were used in the current study. Beginning on PND 1 and continuing daily through weaning on PND 21, all offspring/litter were weighed and gavaged daily with the same dose their dam had received. As there may be variable levels of BPA in the milk (Deceuninck et al., 2015; Doerge et al., 2011; Doerge et al., 2010b; Kurebayashi et al., 2005; Tateoka, 2014; Zimmers et al., 2014), direct dosing of each pup was performed to ensure that all animals/litter received the same designated dose. Previously, we demonstrated that serum corticosterone levels were not elevated in gavaged control rats at weaning relative to same-age rats that did not receive a gavage (Ferguson et al., 2011). Twelve animals/sex/treatment group were assigned to the current study (1/sex/litter). Weaned animals were housed 2 to 3 per cage; cagemates were from the same treatment, sex, and age but were not siblings as only 1/sex/litter was used. When no cage-mate was available, a "companion" animal was used. "Companion" animals were from the same strain, sex, and age as the study animals, but were treatment naïve and were not tested.

For gavage, each rat was removed from its home cage and turned ventral side to the ceiling. The animal care technician gently restrained the rat, as firm restraint was unnecessary. The gavage needle was inserted without the rat exhibiting signs of struggle and the entire procedure took less than 30 seconds.

Weaned animals were tail tattooed with a unique identification number. To ensure that all investigators, including animal care personnel, remained blind to treatment, each treatment group was assigned a random color and number. Only after all data were obtained and deposited into the NIEHS Chemical Effects in Biological Systems (CEBS) databank were the data decoded for treatment group assignment.

Females began daily vaginal lavage procedures six days prior to beginning Barnes Maze testing (i.e., on PND 84 or PND 98) and this procedure continued throughout the study. Vaginal lavages were conducted a minimum of 3.5 hours prior to Barnes maze assessments. Males were comparably handled daily for the same duration.

Barnes Maze Testing

Spatial navigation was assessed beginning at PND 90 (for ½ of each cohort) or 104 (for the remaining ½ of each cohort) for a total of 12/sex/treatment group and continued for seven consecutive days using one of two Barnes maze apparatus as described previously (Ferguson et al., 2012; Rosenfeld and

Ferguson, 2014), but with the modifications detailed below. In brief, each maze was constructed of black Plexiglas with a round top (diameter=122 cm) containing 20 equally spaced holes, each 10.5 cm in diameter, at the perimeter. A black false bottom located 3.5 cm below the maze top could be placed in 19 of those holes. Each rat was assigned a specific "escape" hole. Location of this escape hole was balanced across treatments and sex and did not change across any subject's seven test sessions. This escape hole contained a ramp leading to an opaque escape box. There were three large black extra-maze visual cues placed on the surrounding white walls, a horizontal stripe, large circle, and two vertical stripes. The dimensions and distance of each shape from the Barnes maze are depicted in Fig. S1.

As a mildly aversive stimulus, three 500 wattage lights located 66 cm above the center of the maze top that produced approximately 325 lumens of brightness. For comparison, without those aversive stimuli lights, the brightness at the maze top was approximately 151 lumens from the normal overhead fluorescent lights. The animals were habituated to these lighting conditions and the testing room for 30 minutes prior to beginning the Barnes maze experiments. This type of stimulus has been successfully used without any ill effects in other studies with SD rats (Barrett et al., 2009a; Ferguson et al., 2012; Morel et al., 2015). The testing procedure followed those described previously for rats (Rosenfeld and Ferguson, 2014). Briefly, home cages were moved to the test room at 11:00 hrs (i.e., approximately 30 minutes prior to testing) for habituation. On the first test day, each rat was gently placed into the escape box, which was then covered for 2 minutes. After that 2-minute habituation period, the rat was placed into an opaque Plexiglas tube (diameter= 27 cm, height=23 cm) located in center of the maze. The tube was slowly lifted and the rat was allowed 300 seconds to locate the escape box. If the rat did not locate and enter the escape box during that allotted time, it was gently guided to and allowed to remain in the escape box for 15 seconds prior to being returned to its home cage. If the rat located the escape box, it was also allowed 15 seconds inside before being returned to its home cage. On the six subsequent test days, the same procedure was followed except that the rat was not provided the 2-minute habituation period inside the escape box prior to being placed in the opaque Plexiglas tube at the center of the maze. The maze apparatus was cleaned with 70% ethanol between sessions to remove potential odors. Each subject was assessed for one session/day and each session was videotaped with a 600 line true day night camera with mechanical IR cut filter (Arm Electronics model C600DN2, Roseville, CA) that was interfaced with a computer. During each session, the experimenter measured the latency to locate the escape box with a stopwatch. Thus, those animals that solved the maze quicker than others (i.e., located the escape box faster) have a decreased latency time. These procedures were performed in the Division

of Neurotoxicology at the NCTR/FDA in Jefferson, AR. Videorecordings were later analyzed by Dr. Rosenfeld's laboratory.

Videorecordings were analyzed with Topscan software (Cleversys Inc., Reston, Virginia). By using a three-point tracking system, sniffing directed at a hole was categorized as sniffing at a correct (i.e., the escape hole) or an incorrect hole. The endpoint of sniffing at an incorrect hole was used to define "error rate". Additional endpoints for each session included: latency (seconds) to locate the escape box, total distance traveled (mm), velocity (mm/second), and based on the tracking analysis, search strategy was categorized as random, serial, or direct as defined previously (Jasarevic, 2012; Jasarevic et al., 2011; Jasarevic et al., 2013; Williams et al., 2013). The researchers' categorization of each rat's search strategy was done blind to treatment.

Serum Testosterone Concentrations

Blood was collected after the animals completed three additional behavioral tests measuring locomotor and anxiety assessments; (Rebuli et al., 2015), which were conducted following the Barnes Maze assessments. Blood was collected from males on PND 101 (for ½ of each cohort) or PND 115 (for the remaining ½) and from females in estrus between PND 101–107 (for ½ of each cohort) or PND 115–119 (for the remaining ½). Terminal blood was collected by cardiac puncture and serum prepared. Serum concentrations of total testosterone were measured in duplicate aliquots of ether extracted samples (500 µl), by published methods (Jasarevic et al., 2011; Rosenfeld et al., 2000), using a solid phase [125I]-RIA as per the manufacturer's instructions (TKTT2, Coat-a-Count, Siemens Healthcare Diagnostics, Tarrytown, NY).

Statistical Analyses

Barnes Maze Analyses

Prior to analyzing the data, the investigators agreed upon criteria (listed in Table S1) for excluding individual sessions. These criteria were universally applied across all groups to ensure that no bias was introduced at the outset. A total of 120 rats (n=60/sex) was tested for seven consecutive daily test sessions. Of those, 11 or 1.3% of the sessions were excluded from analysis. A P value ≤ 0.05 was considered significant for all parameters measured.

The data were analyzed using statistical analyses methods previously described (Jasarevic, 2012; Jasarevic et al., 2011; Jasarevic et al., 2013; Williams et al., 2013). A complete randomized design (CRD) split plot in space and time was used to analyze the distance traveled, velocity, incidences of sniffing incorrect holes (error rate), and search strategy, as detailed by (Steel, 1996). The litter was the statistical unit. The denominator of F for testing sex and treatment * sex was litter within treatment * sex (denominator df, den df = 55). The sex is considered the space variable as described in the analyses above. The denominator of F for testing day and treatment * day was litter within day and treatment * day (den df = 330). Day was considered the time variable as described in the analyses above. All other interactions were tested using the residual mean square (den df = 317). These variables were analyzed using the PROC GLIMMIX procedure SAS (Version 9.2, SAS Institute, Cary, NC). Normality tests indicated that total distance traveled, velocity, and incidences of sniffing incorrect holes deviated from normal distribution. Therefore, the residuals were assessed and the actual distribution for each variable was determined, which was applied to the PROC GLIMMIX model in the "DIST" statement. The distribution pattern for velocity and incidences of sniffing incorrect holes was lognormal and search strategy was binary. The total distance traveled was based on ranked data (Conover, 1981). The graphs were generated from the actual data, but the statistical differences are based on the actual distribution for each variable, as listed above. Fisher's protected Least Significant Difference (LSD) was used to determine mean differences, on a per comparison basis. Data are shown and described as the mean ± standard error of the mean. For each significant pairwise comparison, a Cohen's d ([X1 − X2]/ pooled standard deviation) was also determined.

Latency data (as determined by the experimenter and the software program) were analyzed by using a Proportional Hazard Ratio (PROC PHREG in SAS, Version 9.2, SAS Institute). This analysis adjusts for right-censoring (defined here as not locating the escape box within the allotted time) while still accommodating the study design. Effects are reported as a hazard ratio that represents the odds of a subject in a treatment group finding the escape box compared to the other groups tested. A significant result indicates the odds are not 1:1. A result greater than 1 indicates the test group finds the escape box more quickly than all other study groups. A result smaller than 1 indicates that the test group finds the escape box more slowly compared to the other study groups. The litter was again used as the denominator of F for treatment, sex, day, and all possible interactions. These data are reported as the 95% lower confidence limit, mean, and 95% upper confidence limit.

Search strategy (random, serial, or direct) data were analyzed using a generalized mixed-effect model (GLMM) with PROC GLIMMIX (Version 9.2, SAS Institute, Cary, NC). A binomial distribution analysis with PROC GLIMMIX using the same model as above was used to compare the two less efficient strategies (i.e., random and serial) to the more efficient search strategy (i.e., direct). These subsets were grouped at the time of data analysis. The same split plot space and time was applied using a link = logit and a distribution = binomial. The mean separation technique was Fisher's LSD by using average logits. Tabled data were converted to probabilities, which is the probability of employing one of the less efficient search strategies compared to the more efficient direct search strategy.

Serum Testosterone Concentrations

The data were analyzed by sex using a one-way, nonparametric ANOVA on the ranks of the testosterone levels across treatment groups. Table S2 shows the number of observations, and the number of values that were below the detection limit (BLD), by treatment group and sex. A large majority of values were BLD for females; however, this was not the case for males, where no or very few across all treatment groups were BLD. Excluding valid measurements which were BLD would likely bias the analysis. Thus, those samples that were BLD were assigned ½ of the lowest measurable concentration (i.e., 7.0 ng/dL).

2. MOLECULAR ENDPOINTS: Cheong A, Johnson SA, Howald EC, Ellersieck MR, Camacho L, Lewis SM, Vanlandingham MM, Ying J, Ho S-M, Rosenfeld CS (2018) Hypothalamic & Hippocampal Gene Expression & Methylation Changes in Rats Perinatally Exposed to BPA: A CLARITY-BPA Study. Epigenetics (in press).

Animal husbandry and dosing

Comprehensive details on animal husbandry, treatment, and dosing procedures have been published Heindel et al. (2015). Therefore, only brief details are described here. All animal use and procedures were approved in advance by the NCTR Institutional Animal Care and Use Committee and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited facility. Experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (NRC, 2011). The animal rooms for breeding, gestation, and pre- weaning housing were maintained at 23 ± 3°C with a humidity level of 50 ± 20%, and a 12 hour:12 hour light/dark cycle with lights on at 6:00. After weaning on postnatal day (PND) 21, the light cycle was changed to a reverse cycle (light on at 11:00; off at 23:00). A low phytoestrogen diet (5K96 verified casein diet 10 IF, round

pellets, γ-irradiated, Test Diets, Purina Mills, Richmond, IN), and Millipore-filtered water in glass water bottles were provided ad libitum. Housing cages were polysulfone with microisolator tops (Ancare Corp., Bellmore, NY) and contained hardwood chip bedding. Diet was tested for BPA, genistein, daidzein, zearalenone, and coumesterol, and drinking water, cage, and bedding extracts were tested for BPA. All lots of diet had BPA levels below 5 ppb and none of the other materials tests had levels of BPA detectable above the level of the average analytical blanks. Breeder male and female Sprague-Dawley (SD) rats (i.e., F_0) from the NCTR breeding colony were placed in study housing conditions at weaning on PND 21.

Approximately two weeks prior to mating, females were assigned to one of three treatment groups (vehicle control 2,500 µg/kg/day BPA (BPA), or 0.5 µg/kg/day ethinyl estradiol (EE)) based on body weight ranking to produce approximately equal mean body weights in each group. Males were randomly mated with females with the stipulation that no sibling or first cousin pairing was permitted. Breeding occurred in five "loads" or "cohorts", each spaced four weeks apart. Offspring from the last two breedings (i.e., loads 4 and 5) were used for the behavioral studies reported previously (Johnson et al., 2016; Rebuli et al., 2015) and for the molecular assessments described here.

While internal concentrations of BPA were not measured in the current studies, they were assessed previously in a comparable study that tested the effects of BPA in the same animal model, dosing regimen (i.e., daily oral gavage of dams from GD 6 through start of parturition, and direct dosing of pups from PND 1), and similar dosing formulations (0.3% CMC as vehicle; BPA and EE doses in this previous study approached or matched those included in the current CLARITY-BPA study) (Churchwell et al, 2014). Based on this prior study, the predicted maximum circulating levels of aglycone BPA and EE for rats tested in the current study would be: BPA: ~ 200 nM at PND 4 and ~ 20 nM at PND 21 and EE:~ 300 pM at PND 4 and ~20 pM at PND 21. The lower levels of aglycone BPA in older animals are due to maturation of the metabolic capacity that resulted in more efficient conjugation of BPA and EE at PND 21 than PND 4 (Doerge et al., 2010). Since the dosing was stopped at PND 21, the levels of BPA or EE at the time of these molecular assessments at adulthood would be undetectable.

Dams were considered pregnant when a sperm plug or a sperm-positive vaginal cytology was observed [mating day = gestational day (GD 0)]. Beginning on GD 6, dams were dosed by daily gavage with 0.3% carboxymethylcellulose (CMC or vehicle), 2.5 µg BPA/kg bw/day, 25 µg BPA/kg bw/day, 2500 µg BPA/kg bw/day, or 0.5 µg EE/kg bw/day. These BPA doses were selected to provide low, middle, and upper levels of exposure and are below the no-observed-adverse-effect level (NOAEL) of 5 mg/kg bw/day as

detailed in Tyl et al. (2002, 2008). These doses are also within the dose range used in prior studies conducted at the NCTR with the same animal model (Johnson et al, 2016; Rebuli et al, 2015; Cao et al., 2013). In particular, our previous study reported that behavioral differences were observed only among offspring with exposure to the dose of 2500 µg BPA/kg bw/day (Johnson et al., 2016). Thus, we focused on this BPA dose in the current study. The materials used for dose formulation were BPA (CAS # 80-05-7, TCI America, Portland, OR; catalogue # B0494, Lot # 111909/AOHOK [air-milled], ≥ 99.9 purity), EE (CAS # 57-63-6, Sigma-Aldrich, St. Louis, MO; catalogue # E4876, Lot # 071M1492V, > 99% purity), and CMC (Sigma-Aldrich; catalogue # C5013, Lot # 041M0105V). Dams were dosed by daily gavage at a volume of 5 ml/kg bw using a modified Hamilton Microlab® ML511C programmable 115V pump (Hamilton Co., Reno, NV; Lewis et al., 2010). No treatment occurred on the day of parturition, typically GD 22. On the day after birth, (i.e., PND 1), litters were randomly culled to 5/sex where possible and identified with a distinguishable paw tattoo. Only offspring from litters born between GD 20-26 and containing at least 3/sex, a minimum of 9 live pups, and with a difference of number of born male and females pups not greater than 5 were used in the current study. Beginning on PND 1 and continuing daily through weaning on PND 21, all offspring/litter were weighed and dosed by daily gavage with the same dose their dam had received. Twelve animals/sex/treatment group were assigned to the current study (1/sex/litter). Weaned animals were housed 2 to 3 per cage; cage-mates were from the same treatment, sex, and age but were not siblings as only 1/sex/litter was used. When no cage-mate was available, a "companion" animal was used. "Companion" animals were from the same strain, sex, and age as the study animals, but were treatment naïve and were not tested.

Brain collection and processing

Upon completion of the behavior assessments, animals were slightly sedated with carbon dioxide and exsanguinated by cardiac puncture. Males were sacrificed on PND 101 (for ½ of each cohort) or PND 115 (for the remaining ½) and females were sacrificed in estrus between PND 101-107 (for ½ of each cohort) or PND 115-119 (for the remaining ½). Brains were removed from the cranium and rapidly frozen in dry ice, with great care to maintain their morphology. The frozen brains were stored at -80°C until being shipped on dry ice to the Rosenfeld laboratory at the University of Missouri for further processing. The hippocampal and hypothalamic regions were micro-punched on dry ice similarly to PND 1 rat sections detailed in Arumbula et al. (2016) with the exceptions detailed below, and a rat brain atlas (Paxinos, 2013) served as a guide for the two brain regions. For the hypothalamus, a Harris Micro-Punch 2mm in

diameter and 1mm in depth (Catalogue # 15093, Ted Pella, Redding, CA, USA) was used to obtain 2 sequential punches that spanned the rostral to caudal medial regions of this brain region. For the hippocampus, a Harris Micro-Punch 1.0 mm in diameter and 1mm in depth (Catalogue # 15091, Ted Pella) was used to obtain two punches that spanned the rostro-dorsal to caudo-ventral regions and thereby, sample from both the CA1 and CA3 nuclei. All tissues were coded so that the data collection was done blinded to treatment level. Raw data were deposited into the NIEHS Chemical Effects in Biological Systems (CEBS) databank. The initial gene expression data were collected blinded to treatment group, but the identity of the samples was decoded to design the follow-up experiments, including the DNA methylation analysis. For these studies, males and females in the BPA 2500, EE, and vehicle control groups were examined. The BPA 2500 male and female groups were selected for this current study as it was previously determined that females in this group showed the most significant behavioral changes when tested in the Barnes maze (Johnson et al., 2016). Five replicates for each brain region, sex, and group were tested in the current study, as this number of replicates was tested in the previous CLARITY-BPA rat study that showed gene expression differences the hippocampus and hypothalamus at PND 1 (Arumbulua et al., 2016) and in the hypothalamus of California mice (*Peromyscus californicus*) (Johnson et al., 2015).

Gene expression analysis

DNA and total RNA were isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Catalogue #80224; Qiagen, Hilden, Germany). RNA was quantified using a spectrophotometer (Nanodrop 2000, ThermoFisher Scientific). DNA and RNA extracted from rat hypothalamus and hippocampus (thirty samples per brain sub-region, including five males and five females per dose group) was used for the gene expression analyses by quantitative real-time polymerase chain reaction (qPCR). Total RNA was reverse transcribed to complementary DNA (cDNA) with SuperScript III Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA, USA), which included a DNase treatment step. The QuantiTect SYBR Green PCR kit (Qiagen) was used for the qPCR reactions, which were performed with a 7500 Realtime PCR system (ThermoFisher Scientific). The sequences of the primers used in the qPCR analyses are listed in Supplemental Table S3. The nucleotide sequence of the test genes was imported into the free Primer 3 (version 4.0.0, http://primer3.ut.ee/) software to create the primers listed in Supplemental Table S3. Primers were ordered from Integrated DNA Technologies (IDT, Coralville, IA). All primer sequences were validated beforehand to ensure that only a single product was obtained and the

amplicon was the correct size. We also validated the primers based on obtained dissociation curves. For gene expression analysis, the same amount of cDNA (47.6 ng) was used per qPCR reaction and the expression levels of the target genes were normalized against that of an internal reference gene (*Rpl19*). Each sample was run in duplicate and a no template control was included in each assay; the latter showed no amplification in all cases. The cycling conditions were: 50^{o}C (2 minutes) + 95^{o}C (15 minutes) + [94^{o}C (15 seconds)+56^{o}C (35 seconds)+72^{o}C (36 seconds)] X 40 cycles.

Treatment of NbE-1 and AIT cells with 5-aza-cytidine

To validate that the expression of select genes are regulated by DNA methylation, a cell-based analysis was performed. Briefly, an immortalized normal rat prostatic epithelial cell line NbE-1 80 and a tumorigenic prostate epithelial cell line AIT 81 were treated with 0.1 % dimethylsulfoxide (DMSO; Sigma) as vehicle control, 0.5 µM or 1.0 µM 5-aza-2-deoxycytidine (5-aza; Tocris), a DNA methylation inhibitor, every two days for eight days as previously described (Ho et al., 2006; Tang et al., 2012). RNA was extracted from the cell lysates using TRIzol reagent (Invitrogen-ThermoFisher Scientific). Total RNA (1 µg) was DNase treated (Promega) and reversed transcribed for qPCR analysis as described in Cheong et al. (2016).

Bisulfite sequencing analysis

Ten matched rat hypothalamus and hippocampus samples, including five males and five females per treatment group, were used for bisulfite sequencing analysis. These samples were collected from the same animals that were selected for gene expression analyses. In brief, three hundred ng of genomic DNA was bisulfite converted using EZ DNA methylation kit (ZymoResearch) according to the manufacturer's protocol as described previously 66. Bisulfite polymerase chain reaction was performed using 3 µl of the bisulfite converted DNA, Platinum Taq DNA polymerase (Invitrogen), and gene specific primers in a 20 µl reaction volume based on the manufacturer's protocol. Gene-specific primers for bisulfite sequencing were designed using MethPrimer (http://www.urogene.org/methprimer/) with reference to the predicted CpG island as indicated in UCSC Genome Browser (http://wwgenome/ucsc.edu/), and are tabulated in Supplemental Table S3. The size and location of the amplicon generated by each primer-pair in the specific gene (*Bdnf*, Figure 2; *Dnmt3b*, Supplemental Figure S4; and *Esr1*, Supplemental Figure S5) were illustrated (see Results section). The amplicons were

resolved in an agarose gel, purified using GenElute Gel Extraction Kit (Sigma), and cloned into pGEM-T easy vector (Promega) according to the manufacturer's protocol. Recombinant plasmids formed from a single One Shot Top 10 E coli colony (~5-7 colonies per sample) were selected and amplified using TempliPhi DNA amplification kit (GE Healthcare) and sequenced by Macrogen USA (Macrogen Maryland). The methylation status of each CpG site was analyzed using Quantification tool for Methylation Analysis (http://quma.cdb.riken.jp/).

Statistical analysis

Numerical outcome measures are expressed as mean ± SEM. Since some male/female pairs were siblings (Supplemental Table S4), a random effect was added in the statistical models to account for between sibling correlation or within cluster effect and ensure more precise and robust estimates of means and SEMs. Target gene expression was adjusted for internal control gene *Rpl19*. Expression levels in the treatment group relative to that in the vehicle control group were calculated using the $2^{-\Delta\Delta Ct}$ method (Rao et al., 2013). For samples with the expression level of an individual gene being undetectable by qPCR, these samples were excluded in the gene-specific expression and correlation analyses. Out of 30 hypothalamic samples, one male sample was excluded from the EE group in the expression level analyses for *Esr1*. For the hippocampus, the following samples were excluded in the expression analyses for genes *Esr2*, *Esr1*, and *Otr*: (1) two females in the vehicle group for *Esr2*, (2) two females in the vehicle group and one male and one female in the BPA group for *Esr1*, and (3) three females and two males in the vehicle group, one female and three males in the EE group, and two males in the BPA group for *Otr*. Since the cycle threshold values for the endogenous *Rpl19* of the excluded samples were comparable to the other samples, they were included in the expression analyses for the other genes (*Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Avp*, *Ar*, *Oxt*, and *Bdnf*).

All numerical variables were inspected for central tendency, dispersion, skewness and outliers before performing formal statistical analyses. Since all variables showed no concerns of severe skewness of the distributions, parametric statistical models where the variables were not transformed were employed. The box plots of the variables did show some mild outliers (i.e., the outliers were above or below 1.5IQR of upper and lower quartiles, but within 3IQR of the upper and lower quartiles, IQR=inter quartile range). Hence, for each variable, two analyses were conducted- with and without removing outliers to test the sensitivity of the findings to the outliers. Since all results showed no changes of conclusion using either method, only the results from full data (hence with outliers) are presented in the current work.

For each numerical variable at each brain region, its association to the fixed effect of group (vehicle vs. BPA vs. EE) was assessed using a mixed effect model. Since some litters had more than one offspring represented in the current study, which is considered a random effect, these litters were used to account for within litter (or between sibling) correlation. In addition, sex was used to adjust for the association of interest in the model. From each mixed effect model, post hoc means of the numerical dependent variable were estimated and compared between groups. Each random effect model was also compared to an alternative model without considering the within litter correlation. The results from the alternative methods showed agreements to the mixed effect models in post hoc means and standard errors for each group, and thus, the results from the mixed effect models were used in the current study. All statistical tests were performed using SAS 9.4 software (SAS, Cary, NC). P-values < 0.05 were considered statistically significant. Bisulfite sequencing analyses was performed on ~5-7 individual colonies per sample, with five samples per sex per group. Two-way ANOVA and Tukey's multiple comparison tests were used to compare among treatment groups. $P<0.05$ was considered as statistically significant.

Correlation analyses

By using Spearman correlation analyses, the gene expression (based on log 2 ΔCT values to permit inclusion of the vehicle control group in these assessments) and DNA methylation data (expressed in log) were correlated to each other and the previously reported spatial learning and memory results from the same rats (Johnson et al., 2016).

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