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Effects of Developmental Exposure to Bisphenol A on Spatial Navigational Learning and Memory in Rats: A CLARITY-BPA Study

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Abstract

Bisphenol A (BPA) is a ubiquitous industrial chemical used in the production of a wide variety of items. Previous studies suggest BPA exposure may result in neuro-disruptive effects; however, data are inconsistent across animal and human studies. As part of the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA), we sought to determine whether female and male rats developmentally exposed to BPA demonstrated later spatial navigational learning and memory deficits. Pregnant NCTR Sprague-Dawley rats were orally dosed from gestational day 6 to parturition, and offspring were directly orally dosed until weaning (postnatal

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day 21). Treatment groups included a vehicle control, three BPA doses (2.5 µg/kg/day-[2.5], 25 µg/kg/day-[25], and 2500 µg/kg/day-[2500]) and a 0.5 µg/kg/day ethinyl estradiol (EE)-reference estrogen dose. At adulthood, 1/sex/litter was tested for seven days in the Barnes maze. The 2500 BPA group sniffed more incorrect holes on day 7 than those in the control, 2.5 BPA, and EE groups. The 2500 BPA females were less likely than control females to locate the escape box in the allotted time (P value= 0.04). Although 2.5 BPA females exhibited a prolonged latency, the effect did not reach significance (P value = 0.06), whereas 2.5 BPA males showed improved latency compared to control males (P value = 0.04), although the significance of this result is uncertain. No differences in serum testosterone concentration were detected in any male or female treatment groups. Current findings suggest developmental exposure of rats to BPA may disrupt aspects of spatial navigational learning and memory.

Keywords

Endocrine Disrupting Chemicals; Estrogens; DOHaD; Rodents; Barnes Maze; Behavioral Testing; Testosterone

Introduction

Bisphenol A (BPA) is a mass produced industrial chemical (Environment Canada, 2008; Galloway et al., 2010), with production reported to be approximately 15 billion pounds in 2013 (GrandViewResearch, 2014; Vandenberg, 2013). BPA is present in a wide variety of commonly used products and applications, including polycarbonate plastics, metal food can linings, dental sealants, thermal receipt paper, and many other items that are not currently required to be labeled to contain BPA, although the American Medical Association made such a recommendation at its 2011 meeting (Association). The widespread prevalence of this chemical has resulted in chronic exposure of humans, including pregnant women, and non-human animals (Bhandari et al., 2015; Braun et al., 2011; Calafat et al., 2005; Vandenberg, 2013). The estimated median daily intake for the overall US population is ~ 34 ng BPA/kg bw/day (Lakind and Naiman, 2011).

In 2008, the National Toxicology Program (NTP) determined there was “*some concern for effects on the brain, behavior, and prostate gland in fetuses, infants, and children at current human exposures to bisphenol A*”, but that BPA exposure from food contact materials was below a level that might cause adverse health effects and that it was safe overall, even for infants and young children (NTP, 2008). By contrast, the Chapel Hill Report forewarned of negative, and potentially irreversible, outcomes in humans (Vandenberg et al., 2009; vom Saal et al., 2007). Rodent model and human studies show that BPA can be transferred across the placenta (Balakrishnan et al., 2010; Ikezuki et al., 2002; Kawamoto et al., 2007; Nishikawa et al., 2010) and through the milk (Deceuninck et al., 2015; Kurebayashi et al., 2005; Tateoka, 2014; Zimmers et al., 2014), although not all studies agree that this is a substantial route of exposure (Doerge et al., 2011; Doerge et al., 2010b). Fetal rodents possess limited ability to metabolize BPA (Doerge et al., 2011; Doerge et al., 2010a; Ikezuki et al., 2002; Kawamoto et al., 2007; Nishikawa et al., 2010), and therefore higher internal concentrations of bioactive BPA may result compared to adults. If developing organisms are

more susceptible to the potential adverse effects of BPA, perinatal exposure could result in later development of diseases, a hypothesis known as the developmental origin of health and disease.

BPA exposure, especially during the perinatal period, may result in neuroendocrine disruption (reviewed in (Leon-Olea et al., 2014)) and ultimately compromise the normal organizational and activational effects of endogenous steroid hormones (Arnold and Breedlove, 1985; Morris et al., 2004; Phoenix et al., 1959). Spatial navigational learning and memory is programmed by early exposure to endogenous sex steroid hormones and requires a later surge in adulthood for the normal elaboration of these behaviors, with males tending to exhibit better performance in a variety of species, including humans (Galea et al., 1995; Gaulin et al., 1990; Gaulin, 1992; Jasarevic, 2012; Simpson and Kelly, 2012; Williams et al., 1990). As an endocrine disrupting chemical (EDC) that can bind and activate estrogen receptors, as well as other steroidogenic and non-steroidogenic receptors (Lee et al., 2003; Vandenberg et al., 2009; Wetherill et al., 2002; Xu et al., 2005; Zoeller et al., 2005), BPA exposure may negatively impact this cognitive process. Several assessments have been developed that specifically measure spatial navigational learning and memory in rodents, including the Barnes maze, Morris water maze, radial arm maze, and appetite-motivated maze tests (Barnes, 1979; Kuwahara et al., 2014; Morris, 1984; Olton, 1976; Sharma et al., 2010).

Conflicting results, however, have been obtained using such assessments in laboratory rodents exposed to BPA. Some reports have suggested that BPA exposure impairs spatial learning and memory (Diaz Weinstein et al., 2013; Eilam-Stock et al., 2012; Goncalves et al., 2010; Jasarevic et al., 2011; Jasarevic et al., 2013; Kim et al., 2011; Kuwahara et al., 2013; Viberg et al., 2011; Xu et al., 2013); while others suggest minimal or no BPA effects on spatial navigation (Ferguson et al., 2012; Kuwahara et al., 2014; Neese et al., 2013; Ryan and Vandenberg, 2006; Sadowski et al., 2014a; Williams et al., 2013). Differences in species, age at and/or route of exposure, BPA dose, and testing methods may account for those varying results. Here, National Center for Toxicological Research (NCTR) Sprague-Dawley rats were exposed daily during the developmental period to BPA (2.5 µg/kg body weight (bw), 25 µg/kg bw, or 2500 µg/kg bw) and spatial navigation was assessed at adulthood. Concurrent negative (vehicle) and reference estrogen (ethinyl estradiol, EE) controls were also included. This work was performed as part of the larger National Institute of Environmental Health Sciences (NIEHS)/National Toxicology Program (NTP)/Food and Drug Administration (FDA) Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) program, in which other research groups are examining additional behavioral and phenotypic endpoints (Birnbaum et al., 2012; Schug et al., 2013).

Materials and Methods

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 $23 \pm 3^{\circ}\text{C}$ with a humidity level of $50 \pm 20\%$, 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., F_0) 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), $2.5 \mu\text{g}$ BPA/kg bw/day, $25 \mu\text{g}$ BPA/kg bw/day, $2500 \mu\text{g}$ BPA/kg bw/day, or $0.5 \mu\text{g}$ 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 $\pm$ standard error of the mean. For each significant pairwise comparison, a Cohen's d ($[X_1 - X_2]/$ 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 $\frac{1}{2}$ of the lowest measurable concentration (i.e., 7.0 ng/dL).

Results

Distance Traveled and Velocity

For distance traveled, there was a significant interaction of treatment*day ($F(24, 330) = 1.7$, $p = 0.03$). There was no significant two-way interaction for sex*day ($F(6, 317) = 1.9$, $p = 0.09$) nor a significant three-way interaction for treatment*sex*day ($F(24, 317)$, $p = 0.9$). When both sexes in each treatment group were combined and examined across days, significant differences were observed on days 2, 5, and 7 (Fig. 1A). On day 2, EE animals traveled less distance than controls and all BPA treatment groups (p range = 0.01 to 0.05) (Cohen's $d = 0.56$ – 0.72). On day 5, EE animals traveled a longer distance than those in the

2500 BPA group ($p = 0.03$) (Cohen's $d = 0.64$). On day 7, 2.5 BPA animals traveled less distance than 2500 BPA animals ($p = 0.003$) (Cohen's $d = 0.87$).

For velocity (Fig. 1B), there were significant main effects of sex ($F(1, 55) = 28.3$, $p < 0.0001$) and day ($F(6, 330) = 19.3$, $p < 0.0001$). Females were faster than males (51.6 ± 1.9 versus 39.4 ± 1.9 mm/second) (Cohen's $d = 0.89$). The day effect indicated that animals became progressively faster from day 1 to day 7, 33.1 ± 2.2 versus 54.3 ± 2.2 mm/second, respectively ($p < 0.0001$) (Cohen's $d = 0.87$). There were no significant two- or three-way interactions.

Sniffing incorrect hole (error rate) and latency

There were significant two-way interactions for treatment*day ($F(24, 328) = 2.1$, $p = 0.002$) and sex*day ($F(6, 255) = 2.3$, $p = 0.03$), but no significant three-way interaction for treatment*sex*day ($F(24, 255) = 1.0$, $p = 0.4$) in the analysis of frequency of sniffing the incorrect hole. While there were no differences in the number of incorrect holes the control group sniffed on day 1 compared to the other trial days, this group sniffed more incorrect holes on day 2 versus days 4 through 7 (Fig. 1C; p value range = 0.0008 to 0.005) (Cohen's $d = 0.37$ to 0.91). On day 2, EE animals sniffed fewer incorrect holes than controls and all BPA treatment groups (p range = 0.002 to 0.01) (Cohen's $d = 0.82$ –0.93). On day 5, however, EE animals sniffed more incorrect holes than those in the 2.5 and 2500 BPA groups (p range = 0.001 to 0.04) (Cohen's $d = 0.63$ to 0.92). On the last or 7th session, the 2500 BPA animals sniffed more incorrect holes than the control, 2.5 BPA, and EE groups (p range = 0.007 to 0.05) (Cohen's $d = 0.6$ to 0.84). Comparisons of the sex*day interaction indicated that on day 3, females sniffed more incorrect holes than males (Fig. S2, $p = 0.02$) (Cohen's $d = 0.44$).

Analysis of the latency data indicated a significant two-way interaction for treatment*sex ($F = 12.0$, $DF = 4$, $p = 0.02$); however, there were no significant interactions of treatment*day ($F = 28.5$; $DF = 24$, $p = 0.2$) or treatment*sex*day ($F = 16.0$; $DF = 24$, $p = 0.9$). Comparisons of the treatment*sex interaction indicated that 2500 BPA females had an overall longer latency than control females, as evidenced by their reduced likelihood of locating the escape box (Fig. 2A; p value = 0.04). 2.5 BPA females also exhibited a prolonged latency compared to control females, but the effect did not reach statistical significance ($p = 0.06$). In contrast, 2.5 BPA males had a greater likelihood of locating the escape box (i.e., reduced latency) compared to control males (Fig. 2B, $p = 0.04$).

Search Strategy

Binomial analysis comparison of inefficient *versus* direct search strategies revealed only a significant main effect for day ($F(6, 330) = 3.0$, $p = 0.008$). There were no significant interactions for treatment*sex ($F(4, 55) = 1.9$, $p = 0.1$, Fig. 3), treatment*day ($F(24, 330) = 0.8$, $p = 0.7$) or treatment*sex*day ($F(24, 316) = 0.7$, $p = 0.8$). Comparisons of the main effect of day indicated that all subjects were more likely to use the direct search strategy on day 7 than days 1 and 2 (p range = 0.04 to 0.05, Fig. S3), as expected with continued testing.

Serum Testosterone Concentrations

There was no significant main effect of treatment on serum testosterone concentrations. As expected, males had significantly higher concentrations of serum testosterone than females; however, there were no significant effects of treatment in either females ($F(4, 54) = 0.86$, $p = 0.5$) or males ($F(4, 54) = 1.07$, $p = 0.4$). Fig. 4 shows the serum concentration testosterone levels in females and males by treatment group.

Discussion

There were four main goals for this study. The first was to determine the effect of perinatal BPA exposure on spatial navigational learning and memory in NCTR Sprague-Dawley rats. Second, the use of three BPA doses allowed the opportunity to determine potential dose-response curves. Correspondingly, the initial hypothesis was that non-monotonic and low dose effects may be observed, as have been reported previously (Angle et al., 2013; Jones and Watson, 2012; Kim et al., 2014; Vandenberg, 2014; Vandenberg et al., 2013). Third, both sexes were assessed to determine potential sex-dependent effects. Finally, a reference estrogen group (EE) was included to determine potential similarities between EE-induced effects and BPA-induced effects. Additionally, EE was included to verify that the animal model was sensitive to estrogenic activity. With regard to goals one to three, the current study was performed in an attempt to reconcile the conflicting data on the effects of BPA exposure, especially during the perinatal period, and disruption of adult spatial navigational learning and memory in a rodent model. The latency and sniffing incorrect hole data suggest that developmental exposure to BPA compromises certain aspects of this cognitive behavior and that the effects vary by sex and dose concentration.

In females, the highest BPA dose tested here (i.e., 2500 $\mu\text{g/kg bw/day}$) resulted in an overall prolonged latency or decreased likelihood of locating the escape hole (Fig. 2). This difference may partially be due to the fact that on the last trial day, the 2500 BPA animals sniffed more incorrect holes than controls (Fig. 1); however, there was no overall difference in distance traveled or usage of the direct search strategy between these groups (Figs. 1 and 3), as we have observed in prior studies with deer mice developmentally exposed to BPA (Jasarevic et al., 2011; Jasarevic et al., 2013). Therefore, other factors might be contributing to the latency differences. 2.5 BPA females also showed longer latency relative to same-sex controls, although this effect did not reach the statistical threshold of $P = 0.05$ (Fig. 2). These data support the notion that the 2500 BPA dose can lead to neurobehavioral deficits in females. In contrast, males exposed to the lowest dose of BPA showed an overall shorter latency to locate the escape box relative to control males (Fig. 2). Other cognitive behavioral tests might be useful in determining whether the prolonged latency observed in these females equates to generalized learning and memory disruptions.

It might seem that distance traveled and latency would be correlated. However, this is not necessarily the case. For example, a rat can remain still or immobile, resulting in a short distance traveled, but a long latency. On the other hand, a rat can immediately locate the escape box, resulting in a similar short distance traveled, but a short latency. Thus, distance traveled is not the best indicator of Barnes Maze performance. Similarly, it might be thought that velocity and latency should be correlated. However, an animal can exhibit an increased

velocity but still have a prolonged latency while another animal can quickly run to the escape box resulting in increased velocity but short latency. As reported previously, latency and sniffing incorrect holes are thus the best indices of spatial navigational learning and memory (Ferguson et al., 2012; Jasarevic et al., 2011; Jasarevic et al., 2013). Control females improved their performance and learned over the trial period, as evidenced by the fact they sniffed fewer incorrect holes in the later trial days. By day 7, control females sniffed less incorrect holes compared to 2500 BPA females.

In most rodent species and humans, males tend to exhibit superior spatial navigational ability compared to females (Galea et al., 1995; Gaulin et al., 1990; Gaulin, 1992; Jasarevic, 2012; Jasarevic et al., 2011; Jasarevic et al., 2013; Williams et al., 1990). Male deer mice (*Peromyscus maniculatus*) exposed to varying BPA doses exhibited compromised spatial learning and memory. In contrast, females exposed to those same BPA doses exhibited either no change at the higher doses tested or a masculinized response (i.e., improved performance at the lower doses tested) (Jasarevic et al., 2011; Jasarevic et al., 2013). The only sex difference observed in the current study was on day 3, when females sniffed more incorrect holes than males (Fig. S2). While most studies test adult animals, it is difficult to compare the current findings to those in prior BPA studies, where different rodent models, doses, time, and/or routes of exposure were employed. Males of some rodent models might exhibit superior spatial navigational abilities; whereas, females do so in other species. Conversely, there may not be any sex differences in this trait, such as in California mice (*Peromyscus californicus*) (Jasarevic, 2012). Therefore, it is important to examine for potential sex differences in controls of the experimental rodent model being studied.

Prior studies have suggested that female Sprague-Dawley rats subjected to neonatal testosterone treatment exhibit a masculinized or improved spatial navigational performance (Roof and Havens, 1992). Adult gonadectomy of males results in retention deficits that are alleviated by administration of testosterone propionate or estradiol (Locklear and Kritzer, 2014). Females also show greater vulnerability to age-related spatial impairments than males (Barrett et al., 2009b). Those sex differences might be due to declining estradiol concentrations in older female rats, although this has not been directly assessed. Early exposure to BPA may disrupt the normal organizational-activational effects of steroid hormones required to support this behavior in females. Conversely, males exposed to the lowest BPA dose showed improved spatial navigational learning and memory, although the significance of this finding is uncertain. It is not clear why the current findings differ from previous studies. Possible explanations include differences in BPA dose, exposure route (e.g., through the maternal diet vs. maternal and direct gavage), animal model, and other intrinsic factors. Additional tests examining this same behavioral domain would be helpful in verifying the significance of the current results.

Our initial hypothesis was that any behavioral effects observed in the current study may be due to the direct effects of BPA on steroid hormone production or at the level of the hippocampus, the primary brain region governing this cognitive response (Pyter et al., 2005; Pyter et al., 2006; Walton et al., 2011). There is robust evidence that BPA exposure can disrupt hippocampal neural circuitry (Bowman et al., 2014; Elsworth et al., 2013; Hajszan and Leranthe, 2010; Kim et al., 2011; Leranthe et al., 2008a; Leranthe et al., 2008b; Tiwari et

al., 2014; Xu et al., 2013; Xu et al., 2014; Zhang et al., 2014). Conversely, the differential behavioral effects of BPA might be due to effects on other brain regions, such as the prefrontal, orbitofrontal, retrosplenial cortex, medial septum, ventral septum, thalamus, nucleus accumbens, and amygdala (Arias et al., 2014; Czajkowski et al., 2014; Jankowski et al., 2013; Rinaldi et al., 2012), and several other areas which have been shown to be vulnerable to BPA effects (Cao et al., 2014; Cao et al., 2013; Facciolo et al., 2002; Sadowski et al., 2014b). The behavioral disruptions may also originate from sex-dependent epigenetic changes in the brain (Jasarevic et al., 2012; Kundakovic et al., 2013; Yaoi et al., 2008). We are thus currently performing molecular studies with the brain tissues collected from the same animals examined in the current behavioral studies.

It is possible that exposure of the F₀ dams to BPA may have compromised their parental care to their F₁ offspring, the subjects in these behavioral studies. This has been shown to occur in various rodent models where the dams are exposed to BPA and/or related EDC (Boudalia et al., 2014; Cummings et al., 2005; Della Seta et al., 2005; Engell et al., 2006; Johnson et al., 2015; Kundakovic et al., 2013; Palanza et al., 2002). Potential poor parenting received by the BPA-exposed pups may place them at risk for learning and memory deficiencies and other behavioral deficits (Birnie et al., 2013; Dougherty et al., 2013; Parent et al., 2013). We cannot exclude this potential confounder in the current study. To address this possibility, cross-fostering approaches should be considered in follow-up studies (Cox et al., 2010).

In this current study, there were no treatment-related differences in adult serum testosterone concentrations in either males or females, suggesting that disruptions in this sex hormone were not responsible for any observed behavioral differences. With the limited volume of serum available, serum levels of testosterone were measured given that BPA treatment has been suggested to decrease levels in male and female rats (Chen et al., 2014; Lee et al., 2013), although other BPA studies have shown no effects on this steroid hormone (detailed below). Further, testosterone is thought to be one factor that improves spatial navigational performance in males and females (Burkitt et al., 2007; Driscoll et al., 2005; Roof and Havens, 1992). The limited volume of serum remaining after performing the testosterone measurements precluded further assays. However, a prior study with NCTR Sprague-Dawley rats did not show any BPA-related differences in serum estradiol or testosterone concentrations in adult males and females similarly treated with 2.5 and 25.0 µg BPA /kg bw/day (Ferguson et al., 2014). In a 90-day subchronic BPA study that used the NCTR Sprague-Dawley rat (Delclos et al., 2014), the highest BPA doses (100 and 300 mg/kg bw/day) and the two EE doses (0.5 and 5.0 µg/kg bw/day) increased serum estradiol concentrations in PND 90 females, but no significant BPA effects were detected in the treatment groups ranging from 2.5 to 2,700 µg BPA /kg bw/day. Moreover, no BPA dose altered serum testosterone concentrations in those PND 90 males similar to the current results. Deer mice (*Peromyscus maniculatus bairdii*) developmentally exposed to BPA exhibited spatial navigational learning and memory deficits, but no differences in serum testosterone concentrations (Jasarevic et al., 2011). Our current results and previous studies suggest that the observed behavioral differences in the Barnes maze are not due to differences in adult concentrations of testosterone and estradiol. The findings though do not exclude the possibility of differences in these hormones during the pre- and early post-natal periods.

Gene methylation and transcription of candidate genes, including various steroid receptors (*Esr1* and *2*, *Ar*, and *Gr*) will be probed within the hippocampus. Those additional studies aim to discern the potential underpinning mechanisms of the behavioral effects reported here. Performance of those follow-up studies in hippocampal samples obtained from the behaviorally assessed rats will allow a more robust integration and interpretation of the behavioral endpoints. Lastly, an initial assumption was that the neurobehavioral effects of BPA would resemble those of EE, as observed previously in deer mice (Jasarevic et al., 2011; Jasarevic et al., 2013); however, EE treatment did not affect the latency to locate the escape box of either sex in the current study. When EE-treated (5.0 µg/kg bw/day, a dose 10-fold higher than the dose used in the current study) NCTR Sprague-Dawley rats were tested at an earlier age (PND 47 to 50), overall latency was increased in both sexes (Ferguson et al., 2012). Additional doses of EE and testing at various ages may thus be needed to sort out these disparate results. Our current findings though suggest that the BPA-induced behavioral effects may be due to an estrogen receptor-independent manner.

In summary, the current findings indicate that developmental exposure to BPA can disrupt aspects of spatial navigational learning and memory in a sex-dependent manner. While the 2500 BPA, and to a lesser extent the 2.5 BPA dose, prolonged latency in females, the latter dose paradoxically improved responses in males. It remains to be determined if these effects are due to epigenetic/gene expression changes within the hippocampus or other brain regions essential for learning and memory. Ongoing biomolecular studies using the same animals assessed for the endpoints herein should help elucidate the potential mechanisms contributing to the observed behavioral changes and differential responses in males *versus* females.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Male and female rats were pre and postnatally exposed to EE or 1 of 3 BPA doses.
- They were tested as adults (90 to 120 days of age) in the Barnes maze.
- 2500 BPA females were less likely to locate the escape box than control females.
- 2.5 BPA females showed prolonged latency, but effects did not reach significance.
- Early BPA exposure may disrupt aspects of spatial navigational learning and memory.

Fig. 1.

Total distance traveled, velocity, and incidence of sniffing incorrect holes for females and males combined in each treatment group. A) Total distance traveled (mm) (mean $\pm$ SEM). Comparisons of the significant treatment*day interaction indicated that on day 2, EE animals traveled less distance than controls and all treatment groups (p range = 0.01 to 0.05, asterisk). On day 5, EE animals traveled more distance than those in the 2500 BPA group (p = 0.03, delineated by the bracketed line). On day 7, 2.5 BPA animals traveled less distance than 2500 BPA animals (p = 0.003, delineated by the bracketed line). B) Velocity (mm/second) (mean $\pm$ SEM). There were no significant effects detected in the analysis of velocity. C) Incidence of sniffing incorrect holes. Comparisons of the significant treatment*day interaction indicated that on day 2, EE animals sniffed fewer incorrect holes than controls and all other treatment groups (p range = 0.002 to 0.01). On day 5, however, EE animals sniffed more incorrect holes than those in the 2.5 and 2500 BPA groups (p range = 0.001 to 0.04, delineated by bracketed lines). On the last trial day, animals in the 2500 BPA group sniffed more incorrect holes than those in the control, 2.5 BPA, and EE groups (p range = 0.007 to 0.05, delineated by bracketed lines).

Fig. 2.

Overall likelihood for females and males in each treatment group to locate the escape hole. Note that increasing percent likelihood indicates shorter latency. For both panels, the upper, middle, and lower bars represent the percent likelihood of locating the correct escape hole at the 95% upper confidence limit, mean, and 95% lower confidence limit, respectively, of each group to locate the escape hole. Comparisons of the significant two-way interaction for treatment*sex are shown. A) Overall likelihood for females. B) Overall likelihood for males.

Fig. 3.

Comparisons of the use of the efficient direct search strategy *versus* the inefficient search strategies (random and serial) for females (Panel A) and males (Panel B). Yellow= Probability of using an inefficient search strategy (random or serial). Green= Probability of using the direct search strategy. There were no significant effects involving treatment. Thus, while latency (or percent likelihood) differed by treatment and sex, females and males employ the inefficient and direct search strategies at comparable percentages.

Fig. 4. Adult serum testosterone concentrations (mean $\pm$ SEM) in females (A) and males (B). There are no differences in serum testosterone concentrations between any of the female or male treatment groups.