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Bisphenol-A Exposure During Adolescence Leads to Enduring Alterations in Cognition and Dendritic Spine Density in Adult Male and Female Rats

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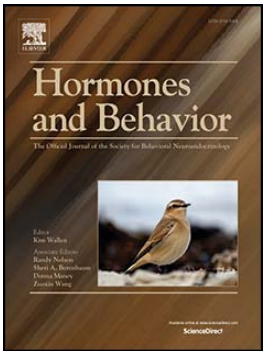

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Bisphenol-A exposure during adolescence leads to enduring alterations in cognition and dendritic spine density in adult male and female rats

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We have previously demonstrated that adolescent exposure of rats to bisphenol-A (BPA), an environmental endocrine disrupter, increases anxiety, impairs spatial memory, and decreases dendritic spine density in the CA1 region of the hippocampus (CA1) and medial prefrontal cortex (mPFC) when measured in adolescence in both sexes. The present study examined whether the behavioral and morphological alterations following BPA exposure during adolescent development are maintained into adulthood. Male and female, adolescent rats received BPA, 40 µg/kg/bodyweight, or control treatments for one week. In adulthood, subjects were tested for anxiety and locomotor activity, spatial memory, non-spatial visual memory, and sucrose preference. Additionally, stress-induced serum corticosterone levels and dendritic spine density in the mPFC and CA1 were measured. BPA-treated males, but not females, had decreased arm visits on the elevated plus maze, but there was no effect on anxiety. Non-spatial memory, object recognition, was also decreased in BPA treated males, but not females. BPA exposure did not alter spatial memory, object placement, but decreased exploration during the tasks in both sexes. No significant group differences in sucrose preference or serum corticosterone levels in response to a stress challenge were found. However, BPA exposure, regardless of sex, significantly decreased spine density of both apical and basal dendrites on pyramidal cells in CA1 but had no effect in the mPFC. Current data are discussed in relation to BPA dependent changes, which were present during adolescence and did, or did not, endure into adulthood. Overall, adolescent BPA exposure, below the current reference safe daily limit set by the U.S.E.P.A., leads to alterations in some behaviors and neuronal morphology that endure into adulthood.

Key terms: bisphenol-A, adolescence, anxiety, memory, exploration, spine density

Bisphenol-A (BPA), is a known endocrine disruptor, documented to have estrogenic, anti- estrogenic, androgenic, and anti-androgenic effects (Negishi et al., 2003; Sohoni and Sumpter, 1998) on various hormone- induced physiological and behavioral phenomena. Detectable levels of BPA have been reported in body fluids of humans and animals (Biedermann et al., 2010; Geens et al., 2011; Rubin, 2011) and, thus, BPA exposure has potential health hazards (Rubin, 2011; Rubin and Soto, 2009; Talsness et al., 2009; vom Saal and Hughes, 2005).

Exposure to BPA during the perinatal period has been shown to reverse or abolish sexual dimorphisms in several brain regions in the rodent (Cao et al., 2014, Kubo et al., 2003, Patisaul et al., 2006, Rubin et al., 2006) and rhesus monkeys (Elsworth et al., 2013). Additionally, perinatal BPA exposure has been shown to reverse sex differences in sweet taste preference, a marker of anhedonia (Katz, 1981), by increasing sucrose preference in adult males and decreasing it in adult females (Xu, X. et al., 2011). In adolescent rats, perinatal BPA exposure increases hyperactivity in males (Ishido et al., 2004; Kiguchi et al., 2008) and eliminates sex differences in both open-field behavior (Fujimoto et al., 2006; Kubo et al., 2003) and the forced swimming test (Fujimoto et al., 2006).

Aggression and anxiety in adult rats is increased following perinatal exposure to BPA (Patisaul and Bateman, 2008; Patisaul et al., 2012), and exploratory behaviors in both adolescent (Fujimoto et al., 2006) and adult rodents are decreased (Farabollini et al., 1999; Goncalves et al., 2010). Importantly, perinatal BPA exposure impairs spatial memory of both male and female adolescent rats (Poimenova et al., 2010). In addition, BPA administration in adulthood alters both object recognition (OR), spatial memory (object placement, OP) and dendritic spine density in male and female rats (Goncalves et al., 2010, Eilam-Stock et al., 2012, Inagaki et al., 2012).

Thus BPA has been demonstrated to have both behavioral and morphologic effects through development and in adulthood.

BPA has also been shown to alter the hypothalamic-pituitary-adrenal axis. Low dose exposure to BPA during the perinatal period increased corticosterone levels under both basal and stress conditions in adolescence (Panagiotidou et al., 2014; Poimenova et al., 2010), altered concentrations of hippocampal glucocorticoid and mineralocorticoid receptors and induced sex differences in plasma corticosterone levels at an earlier developmental age (pre-pubertal) than previously reported (Malendowicz and Mlynarczyk, 1982; Panagiotidou et al., 2014; Poimenova et al., 2010).

Only more recently have investigators turned their attention to the possible effects of BPA during the period of adolescence, which is characterized by hormonal changes, structural alterations in the brain and further programming of some sexually dimorphic behaviors (Juraska et al, 2013). Environmental stressors during this period have also been shown to affect adult behaviors and alter neural plasticity (Holder & Blaustein, 2014). We have previously demonstrated that short term, low-dose BPA adolescent exposure (below the current reference safe daily limit of 50 µg/kg day set by the United States Environmental Protection Agency, [U.S.E.P.A., 1993] increased anxiety on the elevated plus maze (EPM) and open field and impaired spatial memory on the OP task (Diaz-Weinstein et al., 2013). In addition, we found that adolescent BPA exposure increased sucrose preference, and all of these BPA changes occurred independent of sex when tested during adolescence (Diaz-Weinstein et al. 2013). Golgi impregnation studies demonstrated that BPA exposure during adolescence resulted in a decreased dendritic spine density on pyramidal cells in both the mPFC and the CA1 region of the hippocampus during adolescence, an effect that persisted into adulthood (Bowman et al., 2014).

The current study was designed to answer several questions stemming from our previous two studies (Diaz-Weinstein, et al., 2013 and Bowman, et al., 2014) – specifically whether the behavioral and morphological changes observed in adolescence following adolescent BPA are maintained when treated subjects are evaluated at adulthood. We investigated whether BPA exposure during adolescence (postnatal days [PND] 42-49) alters anxiety, cognitive functioning, and sucrose preference in male and female rats tested in adulthood (11 weeks of age). Furthermore, whether adolescent BPA exposure alters serum corticosterone levels in response to a stress challenge was examined. Lastly, the current study determined whether possible differences in adult anxiety and memory following adolescent BPA exposure are accompanied by alterations in spine density in the mPFC and CA1 regions in adult male and female, BPA treated subjects.

Experimental Procedures

Subjects

Thirty two experimentally naïve 5 wk old Sprague Dawley rats (n=16 males, n=16 females) were obtained from Charles River Laboratories (Maryland, USA) and maintained on a 12/12-hr light/dark cycle (lights on 7:00 am). All experimental procedures were approved by the Sacred Heart University Institutional Animal Care and Use Committee and in accordance with the NIH Guide for the Care and Use of Animals. Subjects were double housed according to sex and treatment condition in a common animal colony room, temperature regulated at 21.1°C, and had free access to rat chow (Harlan 2018 Teklad Global) and water (Glass water bottles, Ancare Corporation, Bellmore, NY). All animals were weighed regularly. Table 1 illustrates the overall research design timeline.

Injections

Following a one-week acclimation period during which animals were allowed to adjust to the new housing conditions, male and female adolescent subjects (Andreollo, et al., 2012; Kwekel, et al., 2010; Quinn, 2005), now aged 6 weeks, were randomly assigned to either a control (vehicle only) or experimental group (BPA exposed). BPA (>99% purity grade) was obtained from Sigma-Aldrich Corp (St. Louis, MO). Each rat received a daily subcutaneous injection, 40 µg/kg bodyweight, at the nape of the neck for one week. The BPA was initially dissolved in ethanol for stock solutions and diluted with saline for the injection. While an important consideration when interpreting the current data is the subcutaneous method of BPA administration used compared to an oral route of exposure, the dosing paradigm used in the current study is consistent with previous, related work (Bowman, et al., 2014; Diaz-Weinstein, et al., 2013; for review, Hajszan & Leranth, 2010).

Behavioral Measures

Following injections, rats were allowed to mature to 11wks of age and then underwent a series of behavioral assessments. Open field (OF) testing was conducted first, followed by EPM, OP, OR, and finally sucrose preference testing. Behavioral measures were performed in designated behavioral testing rooms (21.1°C, 43 lumens/square meter) and all behavioral testing occurred between 9:00 and 14:00 hrs. Behavioral measures were obtained in real time by trained laboratory researchers who were blind to treatment assignment consistent with past studies (Bowman & Kelly, 2012; Bowman, et al., 2001, 2002; Diaz-Weinstein, et al., 2013; Eilam-Stock, et al., 2012). In addition, a paired sample t-test was conducted on open field measures scored in real-time versus from video for a group of animals (not otherwise used in the study, n=5) and there are no differences between the two (see Table 2). Furthermore, inter-rater

reliability for real-time open field measurements was assessed using a bivariate correlation. Positive significant correlations were obtained for outer crossings (r(4)=.999, p=.000), inner crossings (r(4)=.991, p=.001), total crossings (r(4)=.998, p=.000), wall climbs (r(4)=.984, p=000), rears (r(4)=.962, p=.009), and grooms (r(4)=.932, p=.021). Behavioral testing occurred during PND 77-90.

Open field

All animals were tested on the OF. In random fashion, rats were placed one at a time in the OF from a common starting area of a 117 x 70 x 45 cm wooden open top box, with the floor divided into 23 cm square grids (5 X 3). Activity in the field was scored for 6 min. Behaviors recorded included the number of outer sector crossings (movements across squares and a measure of general locomotor activity), inner sector crossings (an anxiety-related behavior), total crossings (combined outer and inner sector crossings), rears, wall climbs, and grooms. Both outer and inner sector visits were scored when the subjects' full body, excluding tail, entered a grid.

Elevated Plus Maze

All animals were tested for measures of anxiety using the EPM which is 50.8 cm high and consists of two open arms, 50.8 x 12.7 cm, and two enclosed arms, 50.8 x 12.7 x 40.6 cm, with an open top. The two open arms and the two closed arms are arranged opposite one another around a 10.2 cm square center. Each rat received one trial on the EPM lasting 5 min during which time the number of entries to open and closed arms was recorded. An entry was counted if the animal placed both forelimbs and more than half of their trunk entered the arm. After each animal, the open field was wiped down with Bacdown Detergent Disinfectant spray (Decon Laboratories, Inc., Fisher Scientific) and allowed to dry to minimize olfactory cues. Frequency of

visits to and duration of time spent in open arms was used as an anxiety index, with fewer open arm visits being indicative of increased anxious behavior and total visits (both open and closed arm visits) used as a measure of overall activity.

Object placement and Object recognition trials

Spatial memory was assessed using the OP task. All trials were conducted in an enclosed arena measuring 70 X 70 X 40 cm. The arena was wood with an open top. Three walls were painted grey and one wall was painted black and white striped which provided an intra-maze spatial cue. Trials consisted of a sample trial (T1) and a retention trial (T2), separated by an inter-trial delay. In T1, two identical objects were placed at one end of the open field and amount of time spent exploring the two objects was recorded for 3 min. For T2, one object was moved to a novel location within the field. In T2, the time spent exploring the object at the old location and the new location was recorded for 3 min. Thus, the percentage of time spent with the object in the new location during the total exploration time during T2 is used as an index of object placement performance, (time with new location)/(time with old location + time with new location) x100. This measure is referred to as Ratio. All animals were habituated to the OP test by being exposed to a10 min inter-trial delay (data not shown) and were then tested for object placement memory with a 30 min delay (Figure 2).

Non-spatial, visual memory was assessed using a modified version of the OR task. The testing field was identical to that used for the OP trials with the exception that all four walls were painted grey. The OR task is identical to the OP procedure, except that during T2, the retention trial, one of the original objects is replaced with a new distinct object. Thus, the percentage of time spent with the novel object during the total exploration time during T2 is used as an index

of OR performance. All animals were habituated to the OR test by being exposed to a 10 min inter-trial delay (data not shown) and were then tested for OR with 30 min delay (Figure 3).

During OP and OR trials, exploration was defined as subjects sniffing, whisking, or looking at the object from no more than 2 cm away. The objects used for trials included identical pairs of bottles, cans, ceramic and glass figures. The new location/object was counterbalanced across treatment. The field and all objects were thoroughly cleaned with Bacdown Detergent Disinfectant spray both between T1 and T2 for individual animals and between separate trials for each animal.

Sucrose Consumption

Subjects were given one day of acclimation to the two-bottle choice paradigm prior to sucrose preference testing. During this time, two identical standard water bottles, both containing tap water, were introduced to each cage. Following acclimation, and prior to the sucrose preference test, animals were deprived of food and water access for 24 hours in order to ensure motivation to drink/consume calories from the water bottles. On the test day, animals were again provided with two identical water bottles; however, one contained tap water and the other contained a 10% sucrose solution. The bottles were weighed and placed on cages for 24 hours (tap and sucrose bottle position was switched midway through) at which point the volume consumed for both tap and sucrose bottles was recorded (in grams). Sucrose preference is expressed as the percentage of sucrose consumed (Sucrose intake/Tap + Sucrose [total] intake). Two subjects had consumption levels that were more than two standard deviations below the group mean and were excluded from the data analysis.

Stress challenge

Following behavioral testing, stress was applied using by placing each individual rat in a Plexiglas tube (Harvard Apparatus, item #52029). The Plexiglas restraint tubes were equipped with air holes and an adjustable endplate used to secure the rat within the tube. Once the rats were placed in their respective restraint tube, they were placed in a temperature control room (21.1°C) separate from the main animal colony. Animals remained in the restrainer for 1 hr and were then immediately removed and sacrificed via rapid decapitation (between 12:00 – 1:00 pm).

Golgi Impregnation

Following sacrifice, at 13 weeks of age, brains were removed from subjects and cut into an anterior block (anterior to the optic chiasm) and a posterior block (between the optic chiasm and the brainstem) and placed in solutions provided in the Rapid Golgi Stain Kit (FD NeuroTechnologies, Ellicott City, MD). Golgi impregnation was performed as previously described (Frankfurt et al., 2011; Inagaki et al., 2012). Secondary basal dendrites and tertiary apical dendrites were analyzed blindly from pyramidal cells from the CA1 region of the dorsal hippocampus and layer II/III of the prelimbic portion of the mPFC. Six cells per region/brain were included in the analysis and 6 brains were quantified per group. Neurons in both areas were chosen for analyses as follows: (1) cell bodies and dendrites were well impregnated; (2) dendrites were clearly distinguishable from adjacent cells and continuous. Spines were counted under oil (100x) using a hand counter and dendritic length measured using the Spot Advanced program, version 5.0 Windows (Diagnostic Instruments, Inc.) and a Nikon Eclipse E400 microscope. Spine density was calculated by dividing the number of spines by the length of the dendrite and data expressed as number of spines/ 10 µm dendrite.

Corticosterone measurement

At a sacrifice, trunk blood was collected from all subjects. Samples were centrifuged at 3000g in 4°C for 15-minutes and sera collected. Using a corticosterone ELISA kit (Neogen Corp., Lexington, KY), 100µL of plasma was dissolved in ethyl ether and allowed to evaporate for 48-hours. The ELISA kit used polyclonal rabbit antibodies, had a sensitivity range from 0.05-5.0 ng/ml, and had an inter-assay and intra-assay validation of ≤10%. Samples went through a series of washes and incubations as directed by the kit instructions. Samples and standards (50µL) were assayed in a kit-provided 96-well plate and read in a microplate reader at 650nm. Output was converted into corticosterone levels at ng/ml via equations provided by Neogen Corp.

Data analysis

Data were analyzed using NCSS software (Kaysville, UT, USA). Two-way (sex X treatment) ANOVAs were used to test for group differences. Type I error rate was set at 0.05. Effect sizes were calculated using Eta-squared, η^2, and Fisher's LSD Tests were used for post-hoc analysis, where appropriate.

Results

Open field

A two-way ANOVA (sex X treatment) was used to examine possible group differences in the OF. There were no significant treatment or interaction effects on any of the dependent measures (outer crossings, inner crossings, total crossings, rears, wall climbs, or grooms. $p>.05$, data not shown). For the number of inner sector visits females made more crosses (7.13 ± 1.85)

than males (2.0 $\pm$ .63) regardless of treatment, main effect of sex, $F(1,31)=6.83$, $p=0.014$, $\eta^2 = 0.19$.

Elevated Plus Maze

Visits made during the EPM are shown in Figure 1. A two-way ANOVA (sex X treatment) was used to examine possible group differences in the number of open, closed, and total visits during the 5 min EPM trials. There were no group differences in the percentage of time or frequency to the open arms. Additionally, while there were no main effects on the total number of visits, there was a significant sex X treatment interaction, $F(1,31)=6.72$, $p=0.015$, $\eta^2 = 0.18$. Post hoc testing revealed that BPA exposure decreased total visits in males, but not females. The same interaction, $F(1,31)=6.08$, $p=0.02$, $\eta^2 =0.17$, was observed in the number of visits made to the closed arms. The number of closed arm visits was decreased in BPA treated males, while no group differences were observed for females, regardless of treatment. No significant group differences were observed for the number of visits made to open arms ($p>.05$). Thus, while BPA treatment decreased overall visits of males, no changes in open arm visits in BPA treated subjects indicates no effects on anxiety-like behavior.

Object placement

Figure 2A shows the exploration times during the sample (T1) and retention (T2) trials of the 30 min OP delay. During the 30 min inter-trial delay, there were no group differences in initial exploration times during the sample trial (T1, $p>.05$). During T2 of the 30 min inter-trial delay, BPA treated subjects spent less time exploring (7.44 + 1.48) than controls (12.53 + 1.86) regardless of sex, main effect of treatment $F(1,29)=4.93$, $p=0.035$ $\eta =0.15$. No significant group differences were observed on the percentage of time spent with the object in the new location

(ratio), Figure 2B. Thus, BPA treatment altered exploration but not performance of the spatial memory task.

Object Recognition

Figure 3A shows the exploration times during the sample (T1) and retention (T2) trials of the 30 min inter-trial OR delay. There were no significant group differences in exploration times for either T1 or T2. There was a significant sex X treatment interaction effect on the percent time spent with the novel object during the 30 min inter-trial delay, $F(1,31) = 5.50$, $p=0.026$, $\eta^2 = 0.15$. As shown in Figure 3B, post hoc testing revealed that the percent time spent with the object in the new spatial location was decreased in BPA treated males but not BPA treated females.

Sucrose Preference

Preference for a sucrose solution was measured during a 24 h period and possible group differences were analyzed using two-way ANOVA (sex X treatment). All groups had a preference for the sucrose solution and there were no significant main or interaction effects ($p>.05$), see Table 3 for the descriptive statistics.

Serum Corticosterone

All subjects received a 1 h restraint stress challenge immediately prior to sacrifice and serum corticosterone levels were measured and group differences were analyzed using two-way ANOVA (sex X treatment). There were no significant group differences. While BPA treated subjects had higher corticosterone levels following the stress challenge than controls, this difference was not significant ($p>.05$), see Table 3.

Dendritic Spine Density

CA1

Basal and apical CA1 dendritic spine density was measured in pyramidal cells of control and BPA-treated male and female rats following behavioral testing at 13 weeks of age and data were analyzed by two-way ANOVAs (sex X treatment). Figure 4 is a photomicrograph illustrating Golgi impregnated secondary basal dendrites from CA1 from male BPA treated (top) and control (bottom) rats. Effects of BPA treatment are shown in Figure 5A. Spine density on basal dendrites was decreased by 19% in all BPA treated subjects (9.3 $\pm$ 0.20) compared to controls (11.4 $\pm$ .21), regardless of sex, (main effect of treatment, $F(1,22)=65.10$, $p=0.000001$, $\eta^2 = 0.75$ (changes in males and females shown separately; combined data not shown). No significant sex or interaction effects on basal dendritic spine density were observed.

The same pattern was observed in the spine density of CA1 apical dendrites. Spine density on apical dendrites was decreased by 21% in BPA treated subjects (10.3 $\pm$ 0.20) compared to controls (13.0 $\pm$ 0.20), regardless of sex, $F(1,22)=82.16$, $p=0.000001$, $\eta^2 =0.80$ (Figure 5A changes in males and females shown separately; combined data not shown).

mPFC

The pattern of results in layer ll/lll mPFC pyramidal cells was different than that observed in CA1. As shown in Figure 5B, there were no significant group or interaction differences in spine density on either basal or apical dendritic spine density ($p>0.05$).

Coefficients of Variation

In the current study, female estrous cycle was not tracked. In an effort to better understand the possible contribution of cycle stage to the outcomes reported here, we (1)

calculated a coefficient of variance ratio (CV) for males and females across all dependent measures and (2) tested for homogeneity of variance using the Levene's test of homogeneity of variances (Abdi, 2010; Wallen & Lloyd, 2008), see Table 3. Out of 21 dependent measures, only 3 (inner sector crossings and rears in the open field and the OP ratio) violated the homogeneity of variance assumption as tested by Levene's. However, as shown in Table 4, the CV ratios were higher in males than females. Thus, the difference in variance was due to CV ratios in males, not females, suggesting that estrous cycle did not play a major role in detecting differences between control and BPA treated females or between males and females.

Discussion

The present results extend findings regarding the effects of BPA administration during adolescence. We have previously demonstrated that BPA given during adolescence results in increased anxiety on both the EPM and OF, impaired spatial memory on the OP, and increased sucrose preference in both male and females when tested during adolescence (Diaz-Weinstein, et al., 2013). In this study, we examined the effects of the same dose/timing of BPA administration on behaviors in adulthood in order to determine whether the effects of adolescent BPA exposure are maintained. Interestingly, we found that the effects of adolescent BPA exposure altered some, but not all, parameters examined from adolescence to adulthood. Specifically adolescent administration of BPA did not affect anxiety, spatial memory, sucrose preference or dendritic spine density in mPFC but did effect exploration, object recognition memory and dendritic spine density in CA1.

Data from the present study also demonstrate that adolescent exposure to BPA on anxiety in adulthood differs from that seen in adolescence where BPA exposure increased anxiety in both

males and females (Diaz-Weinstein, et al., 2013). Typically, anxiety is measured on the EPM as decreased exploration of the open arms and/or increased visits to the closed arms (Carobrez & Bertoglio, 2005). Here BPA treated males had decreased visits to the closed arms, which is reflected as an overall decreased activity on the EPM (visits to either open or closed arms). Specifically, BPA treated males are significantly different from male controls and BPA treated, but not control, females. It is hard to determine whether this performance on the EPM of BPA treated males reflects an overall change in anxiety or a change in willingness to explore. It is important to note, however, that BPA did not alter locomotor or anxiety measures on the open field in either males or females, which also differs from what was observed in adolescence (Diaz-Weinstein, et al 2013). It is unclear why exploration was decreased in the EPM, but not OF. One possibility is simple habituation to behavioral testing, but clearly future studies are necessary to examine this. Additionally, previous studies have shown that perinatal exposure to BPA decreased exploratory behaviors in both adolescent (Fujimoto et al., 2006) and adult rodents (Farabollini et al., 1999; Goncalves et al., 2010). The current results are novel in that they indicate that short term low dose exposure to BPA during adolescence leads to lasting changes in adulthood that are sex dependent, with males more vulnerable than females, but that, rather than caused by anxiety , these changes are appear to be related to exploration. Additionally, in the present study we observed significantly higher anxiety levels in males than in females, regardless of treatment, as evidenced by the females making more inner sector visits than males, which is in agreement with previous reports (Zimmerberg and Farley, 1993; Imhof et al, 1993; Beck and Luine, 2002; Bowman et al., 2009).

BPA exposure during adolescence did not impair spatial memory during OP performance during adulthood in this study, which differs from what was seen when tested in adolescence

(Weinstein-Diaz, 2013). It has been demonstrated that perinatal BPA exposure impairs spatial learning and memory on the Morris Water Maze at weaning and young adulthood (Xu, X.H. et al., 2010), and long term BPA exposure impairs spatial memory on the Y-maze in adulthood (Tian et al., 2010). Lastly it was shown that a single low-dose exposure in adulthood impairs object placement performance in males (Eilam-Stock et al., 2012) and females (Inagaki et al., 2012). However, it is important to note that in the current study adolescent BPA exposure decreased adult exploration during T2 of the OP and this reduction in exploration may influence the ability to discriminate between the old and new spatial locations. This effect was seen in both males and females and may contribute the lack of a significant sex difference observed in OP performance; although the more likely explanation for the lack of sex differences on the OP task is the short inter-trial delay (i.e., 30 min) because the male advantage on the OP task is typically only evident following longer inter-trial delays (Luine, 2014). The observation that BPA did not impair spatial memory using the task in the current study is consistent with previous reports that perinatal BPA exposure did not alter spatial memory performance on the radial arm maze in male or female rats during adulthood (Sadowski et al., 2014).

As previously reported, adult performance on the OR task was not influenced by sex (Luine, 2014) but was impaired by adolescent BPA exposure in a sex-dependent manner. BPA did not alter exploration during either T1 or T2 of the OR task, but discrimination between the old and new object following a 30 min inter-trial delay was decreased in male, but not female, BPA treated subjects. Previous studies have shown that prenatal BPA exposure impaired performance on the object recognition task in adulthood (16 weeks at behavioral testing, Goncalves et al., 2010) and that a single low dose exposure in adulthood impairs male performance on the object recognition (Eilam-Stock et al., 2012). The current data demonstrates

a similar impairing effect on OR memory in adulthood following adolescent BPA exposure but also reports a significant sex dependent treatment effect not previously observed in which males, but not females, are affected. Thus, for recognition memory, it appears that adolescent treatment with BPA is associated with impairments of object memory in males, but not females; whereas place memory is not impaired in either sex at adulthood. The neural basis for these effects are unknown but changes in neural morphology may be a contributing factor (see below).

The same dose/administration paradigm of BPA used in the current study decreased dendritic spine density in CA1 and mPFC in behaviorally naïve males and females when measured at 7 and 11 weeks of age, and females had greater dendritic spine density than males in basal dendrites of CA1and the mPFC (Bowman et al, 2014). In the current study, at 13 weeks of age, following behavioral testing, we did not see any sex differences in dendritic spine density in CA1 or the mPFC. Sex differences in dendritic branching (Markham et al., 2005) and in proestrus females as compared to males (Shors et al, 2001) have been reported for CA1. However our results are consistent with several studies which have demonstrated a lack of sex differences in either region of adult (Gould et al, 1990b, Salas-Ramirez et al 2010) and 21 day old rats (Frankfurt et al, 2009) The dendritic spine density data from our two experiments suggests that a sex difference during adolescence is no longer present when measured in adulthood, a result consistent with the later maturation of the prefrontal cortex in males vs. females (Markham et al, 2013).

Treatment with BPA decreased dendritic spine density in CA1 of both males and females at 13 weeks following behavioral testing, indicating that the effect of BPA exposure during adolescence persisted into adulthood. These results are consistent with others that have

demonstrated BPA-induced decreases in spine density in adulthood (Bowman, et al., 2014; Eilam-Stock et al., 2012; Leranth et al., 2008ab; McLusky et al., 2005; Xu, X.H. et al., 2013). In contrast, the effects of adolescent BPA exposure on mPFC were no longer present when measured at 13 weeks of age. It is possible that the behavioral experiences interacted with the effect of adolescent BPA exposure on adult mPFC dendritic spine density. Eilam –Stock (2012) found that a single BPA injection decreased dendritic spine density in both CA1 and mPFC in male rats when measured after behavior but had no effect on spine density when behavior was not tested. However, another intriguing explanation is for the lack of effect of BPA in the mPFC is evidence of a possible recovery effect. This view is supported by the observation that sucrose preference, OP, and anxiety are decreased in adolescents after BPA exposure and unchanged in adults. All of these observations suggest a remarkable plasticity of the adolescent brain. Future studies are necessary to address these possibilities.

It has previously been reported that females have a greater sucrose preference than males (Atchley et al., 2005; Sclafani & Mann, 1987; Valenstein et al., 1967; Wade & Zuker, 1969); however, this sex difference has great variability (Loney et al., 2011) and can depend on other factors such as housing (Hong et al., 2012), testing duration (Sclafani et al. 1987), and solution concentration (Sclafani & Clare, 2004). The current results did not demonstrate a sexually differentiated sucrose preference at 13 weeks of age and show that the effect of adolescent BPA exposure on sucrose preference differs from adolescence to adulthood. Perinatal exposure to BPA has been shown to increase sucrose preference in adult males but decrease it in adult females (Xu, X. et al., 2011). Whereas adolescent BPA exposure increased sucrose preference in adolescents of both sexes (Diaz-Weinstein et al., 2013), in adulthood, BPA had no effect on sucrose preference in either sex. Taken together these findings suggest that perinatal exposure to

BPA may have organizational effects while adolescent exposure to BPA may have activational effects during adolescence that are reversed in adulthood. Moreover, the current results indicate that BPA's effects on adult behaviors seen in this experiment (e.g., exploration) are not related to depression-like symptoms.

Adolescent BPA exposure did not alter corticosterone levels following a restraint stress challenge in adulthood, a result similar to our previous findings at adolescence (Bowman, et al., 2014). Previous studies have shown that prenatal BPA exposure leads to stress-induced increases in corticosterone following stress challenges in adolescence (Panagiotidou et al., 2014; Poimenova et al., 2010) suggesting that BPA interferes with the organization of the stress response/hypothalamic-pituitary-adrenal axis during development. These results suggest that BPA does not exert effects during adolescence that are apparent in adulthood.

Recent research has shown that sexual differentiation of the brain is not limited to the perinatal period but that further sexual differentiation occurs during the period of puberty and adolescence (Juraska et al, 2013; Holder & Blaustein, 2014). Thus, it is possible that the currently described effects of BPA, which can act as an estrogen agonist or antagonist or as an androgen antagonist (Negishi et al., 2003; Sohoni and Sumpter, 1998), may be altering gonadal hormone actions in relation to sexual differentiation and may account for the different behavioral responses on the EPM and OR memory task in adult males vs females following adolescent BPA. The observation that some adolescent BPA effects were not maintained into adulthood is also consistent with the theory that the brain is more sensitive to the effects of hormones during the perinatal period than at adolescence (Schulz et al, 2009). Thus, for example, prenatal BPA treatment impaired adult spatial memory (Xu, X.H. et al., 2010), but adolescent BPA

treatment impaired spatial memory (OP) only during adolescence but not at adulthood. A possible confounding variable in the current study is the state of the estrous cycle in the female rats. Even though BPA has been shown to have no effect on estrous cyclicity itself (Ferguson, et al., 2014), additional studies should assess whether BPA's effects differ across specific estrous cycle days. Future studies should account for estrous cycle stage, and clearly, additional research is necessary to determine the mechanisms responsible for BPA-dependent effects.

In sum, the behavioral effects of BPA are more pronounced in adolescence than adulthood. Also, it appears that adolescent BPA exposure leads to enduring effects in adulthood for some measures (e.g., EPM, OR) that are more prominent in males than females. Because BPA is produced at an estimated rate of 6.5 billion pounds per year and humans are routinely exposed through a variety of sources (e.g., food and beverage containers, as well as environmental and trans-dermal exposure sources), the current findings emphasize the essential need to better understand BPA's effects on behaviors and neural mechanisms underlying these changes, especially with regard to how these effects may vary depending on sex, age of exposure and age of testing.

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Figure Legends.

Figure 1. Visits made during elevated plus maze testing. Data is the average ± SEM number of entries made (open, closed, and total arm entries). All significant effects are $P<0.05$ and significant differences between groups are denoted by *. Significant sex X treatment interactions showed that BPA treated males, but not females, had decreased total visits and decreased visits to the closed arms.

Figure 2. Object placement performance. All significant effects are $P<0.05$ and significant differences between groups are denoted by *. In panel A, data are expressed as the total time spent exploring (in seconds, M + SEM) for both the sample (T1) and retention (T2) trials. BPA treated subjects spent less time exploring during T2 than controls. In panel B, data are expressed as the percent of total T2 time spent with the object in the new location (Mean + SEM). No significant group differences were observed in the ratio. Thus, BPA treatment altered exploration but not performance of the spatial memory task.

Figure 3. Object recognition performance. All significant effects are $P<0.05$ and significant differences between groups are denoted by *. In panel A, data are expressed as the total time spent exploring (in seconds, M + SEM) for both the sample (T1) and retention (T2) trials. There were no group differences in exploration times. In panel B, data are expressed as the percent of total T2 time spent with the object in the new location (Mean + SEM). A significant sex X treatment interaction showed that discrimination between old and new objects was decreased in BPA treated males, but unaffected in females.

Figure 4. Photomicrograph illustrating Golgi impregnated secondary basal dendrites from CA1 pyramidal cells in the dorsal hippocampus from male BPA treated (top) and control (bottom) rats. Taken under oil at 100x. Scale bar = 10µm. Arrows denote spines.

Figure 5. Basal and apical dendritic spine density in CA1 and mPFC. Entries are the average # spines/10 µm $\pm$ SEM. All significant effects are $P<0.05$ and group differences are denoted by *. Panel A shows that adolescent BPA exposure led to decreased spine density on both basal and apical dendrites in CA1. Panel B shows that there were no significant effect of adolescent BPA exposure in mPFC.

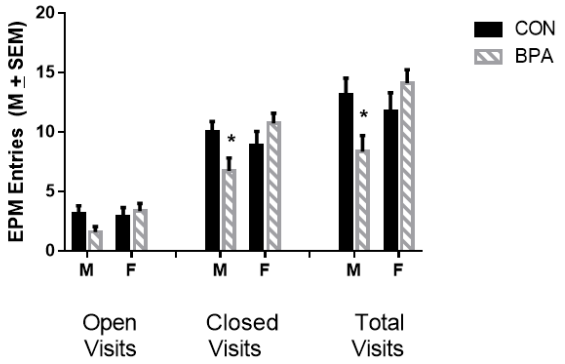

Figure 1

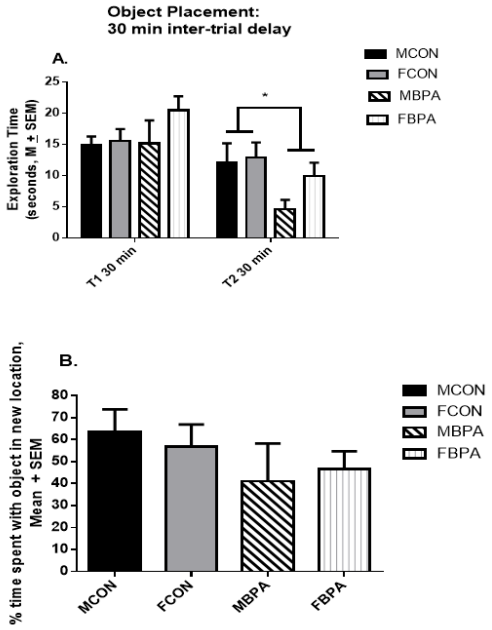

Figure 2

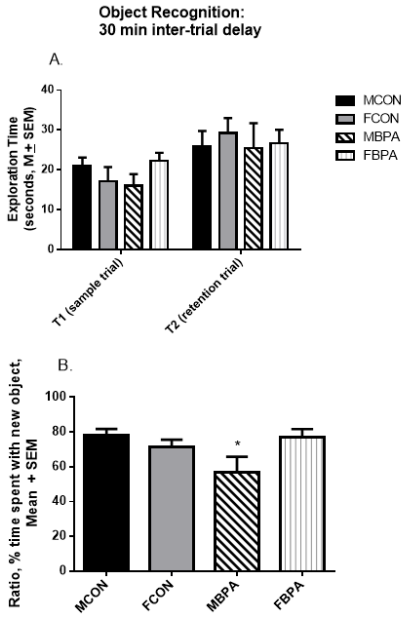

Figure 3

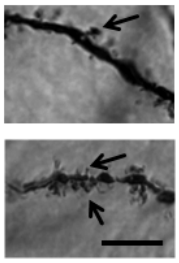

Figure 4

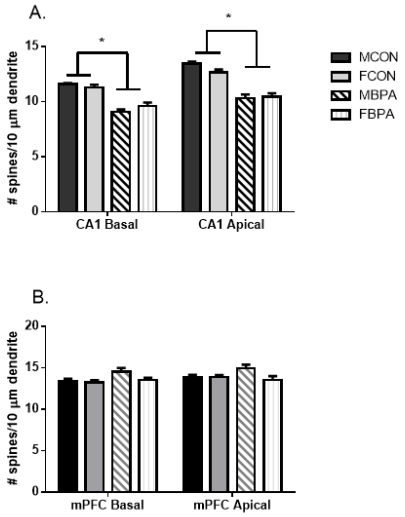

Figure 5

Table 1. Methodological timeline

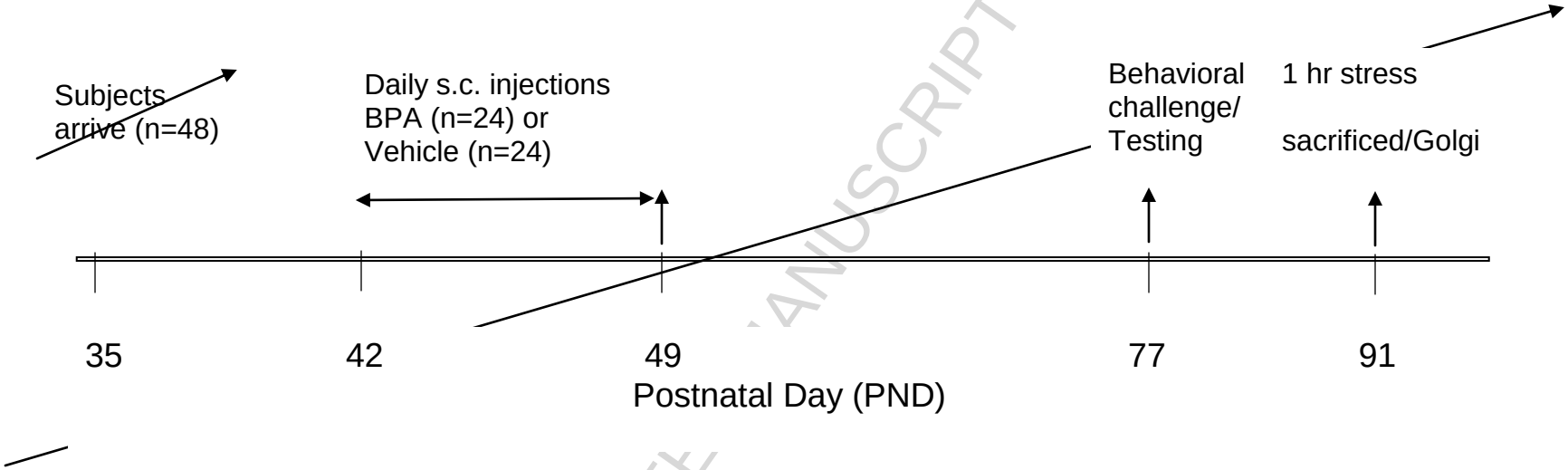

Table 2. Open field behavioral measures scored in real-time versus video.

OF Behavior	Real-time	Video	t-test results
Outer crossings	77.60 ± 9.98	79.00 ± 9.86	t (4) = -0.98, p=0.39
Inner crossings	5.60 ± 1.81	6.00 ± 1.82	t (4) = -1.00, p=0.37
Total crossings	87.60 ± 12.18	91.80 ± 13.45	t (4) = -1.21, p=0.29
Wall climbs	22.20 ± 3.87	21.00 ± 3.71	t (4) = 2.06, p=0.11
Rears	4.20± 2.24	2.80 ± 1.71	t (4) = 0.98, p=0.39
Grooms	1.40 ± 0.51	1.20 ± 0.49	t (4) = 1.00, p=0.37

Note. Data are the M ± SEM for open field measures collected from five rats. There were no statistical differences between the data scored in real-time versus video.

Table 2. BPA exposure did not alter sucrose preference or serum corticosterone levels.

Group	% Sucrose Consumed (grams)	Corticosterone (ng/ml)
Male Control	95.13 ± 1.50	208.46 ± 54.09
Male BPA	97.82 ± 0.67	258.91 ± 31.84
Female Control	94.55 ± 1.65	277.68 ± 50.39
Female BPA	90.96 ± 3.57	323.21 ± 50.36

Note. Data is the Mean + SEM. Exposure to BPA during adolescence did not significantly alter sucrose preference, $F(1,31)=2.04$, $p=0.16$, or stress-induced serum corticosterone levels, $F(1,31)=1.02$, $p=0.32$, compared to control subjects.

Table 3. Homogeneity of variance test results between males and females for all dependent measures.

	Levine's F test	CV females	CV males
EPM:			
Open visits	F (1, 30) = 0.31, p = 0.58	63.84	75.12
Closed visits	F (1, 30) = 0.51, p = 0.48	30.95	38.96
Total visits	F (1, 30) = 0.16, p = 0.69	30.32	41.94
OF:			
Outer crossings	F (1, 30) = 0.81, p = 0.38	49.80	49.79
Inner crossings	*F (1, 30) = 19.36, p = 0.01	104.00	125.00
Total crossings	F (1, 30) = 2.46, p = 0.13	52.34	48.80
Wall climbs	F (1, 30) = 3.76, p = 0.06	72.16	58.13
Rears	*F (1, 30) = 7.38, p = 0.01	165.68	279.36
Grooms	F (1, 30) = 0.73, p = 0.39	137.60	150.00
30 min OP delay:			
T1	F (1, 29) = 0.35, p = 0.56	34.28	52.13
T2	F (1, 29) = 0.01, p = 0.98	57.07	86.26
Ratio	*F (1, 29) = 5.65, p = 0.02	54.97	80.29
30 min OR delay:			
T1	F (1, 30) = 0.04, p = 0.84	42.51	40.48
T2	F (1, 30) = 1.25, p = 0.27	35.17	54.56
Ratio	F (1, 30) = 1.29, p = 0.27	16.98	31.80
Sucrose Preference:			
% Sucrose Consumed	F (1, 30) = 0.02, p = .91	26.68	26.92
Stress Challenge:			
Serum CORT	F (1, 30) = .89, p = 0.35	46.47	53.08

Dendritic Spine Density:			
CA1 basal	F (1, 21) = 2.87, p = 0.11	10.52	13.52
CA1 apical	F (1, 21) = 2.53, p = 0.77	11.43	14.95
mPFC basal	F (1, 21) = 0.80, p = 0.38	5.23	7.79
mPFC apical	F (1, 21) = 0.26, p = 0.13	6.42	7.05

Note: *denotes significant differences in variances as indicated by Levene's test.

Highlights

- Effects of short term low dose BPA adolescent exposure were examined in male and female adult rats.
- Adolescent BPA decreased exploration, but not spatial memory in adulthood.
- Adolescent BPA impaired male, but not female, performance on the object recognition task in adulthood.
- No alterations in sucrose preference or stress-induced corticosterone levels were observed.
- Adolescent BPA decreased CA1, but not mPFC, dendritic spine density in adulthood.