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Bisphenol-A Impairs Memory and Reduces Dendritic Spine Density in Adult Male Rats

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Abstract

Exposure to Bisphenol-A (BPA), an endocrine disruptor used in plastics, occurs in the United States on a daily basis. Recent studies suggest exposure during development causes memory deficits later in life, however the ramifications of exposure in adulthood are unclear. We examined the effects of acute BPA administration (40μg/kg) on memory and synaptic plasticity in adult male rats. BPA significantly impaired both visual and spatial memory and decreased dendritic spine density on pyramidal cells in CA1 and the medial prefrontal cortex (mPFC). Additionally, BPA significantly decreased PSD-95, a synaptic marker, in the hippocampus and increased cytosolic pCREB, a transcription factor, in mPFC. Together, these findings show that a single dose of BPA, below the U.S.E.P.A. reference safe daily limit of 50 ug/kg/day, may block the formation of new memories by interfering with neural plasticity processes in the adult brain.

Keywords

Bisphenol-A; endocrine disruptor; memory; synaptic plasticity

Bisphenol-A (BPA, 4, 4′-isopropylidene-2-diphenol) is a synthetic chemical that is widely used in the manufacture of many different products, such as food and beverage containers (Kang, Kito & Kondo, 2003), baby bottles (Brede, Fjeldal, Skjevrak & Herikstad, 2003) and dental sealants (Suzuki, Ishikawa, Sugiyama, Furuta & Nishimura, 2000; Joskow et al., 2006). It has been well established that BPA, through contact with acidic or basic components, or heat, leaches from containers into the food and beverages we consume. Indeed, detectable levels of BPA have been found in urine (Calafat et al., 2005), blood (Ikezuki, Tsutsumi, Takai, Kamei & Taketani, 2002), breast milk (Kuruto-Niwa, Tateoka, Usuki & Nozawa, 2007) and saliva samples (Joskow et al., 2006) of people and animals. The estrogenic properties of BPA have been known since the 1930s (Dodds & Lawson, 1936), however, since BPA has a low affinity for nuclear estrogen receptors, it was not a source of public concern until relatively recently. Nevertheless, over the last two decades, a growing body of research has indicated that even very low doses of BPA, can rapidly interfere with estrogen and androgen activities, presumably through membrane-bound

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receptors, resulting in many physical and mental alterations (see Hajszan & Leranth, 2010; Wolstenholme, Rissman & Connelly, 2011, for review).

Since both estrogens and androgens have an essential role in memory (see Dohanich, 2002; Luine, 2008, for review), it is important to establish whether BPA alters these processes. Previous studies revealed that chronic exposure to BPA in male and female rats (Carr et al., 2003; Goncalves, Cunha, Barros & Martinez, 2010) and male mice (Xu, Zhang, Wang, Ye, & Luo, 2010) during development, had an antagonizing effect on memory later in life. In addition, an earlier study from our lab (Inagaki, Frankfurt, Friedman & Luine, 2007) demonstrated that BPA has an antagonizing effect on memory of adult female rats. In that study, BPA blocked estrogen-induced memory enhancement in adult ovariectomized (OVX) rats, and that is the only extant data, as of today, about BPA effects on memory in the adult brain.

BPA has been shown to influence synaptic plasticity processes in brain areas involved in memory (e.g., hippocampus and prefrontal cortex). Recent studies have found that BPA can block estrogen-induced increases in spine synapse formation in these areas in adult OVX female rats (MacLusky Hajszan, & Leranth 2005). BPA also blocked androgen-induced spine synapse formation in the CA1 region of the hippocampus and layer II/III of the medial prefrontal cortex (mPFC) in adult gonadectomized male rats and reduced the number of spine synapses in these areas of gonadally intact male rats (Leranth, Szigeti-Buck, Maclusky & Hajszan, 2008b). Similar effects were observed in OVX female monkeys (Leranth, Hajszan, Szigeti-Buck, Bober & MacLusky, 2008a), demonstrating that BPA interference in synaptic plasticity processes is not restricted to rodent models. Thus, BPA may influence memory processes in both rodent and human adults.

Although there has been some progress in understanding BPA's effects on memory over the last decade, there is still much to be clarified. In most of the behavioral studies, BPA was given during development, and none of them, to our knowledge, investigated the effects of BPA when administered to adult male subjects. Additionally, most of these studies examined chronic BPA administration at doses greater than what humans experience. Furthermore, morphological studies to date, which demonstrate the effects of BPA on synaptic plasticity in adult animals, have not linked behavioral results to the morphological ones. Finally, the rapid effects of BPA, and its weak affinity for nuclear gonadal hormone receptors, strongly suggest that these receptors are not involved in the actions of BPA on memory, though the underlying mechanism has yet to be fully elucidated.

The current study is an attempt to answer some of these questions. Using adult male rats, we investigated the effects of acute BPA administration on spatial and visual memory at a dose, 40ug/kg, which is below the U.S.E.P.A. reference safe daily limit of 50 ug/kg/day (U.S.E.P.A., 1993). Furthermore, we investigated whether these effects might be linked to dendritic spine density in the CA1 area of the hippocampus and layer II/III of the mPFC. These brain areas were chosen because they are critical for memory function (Diamond, Fleshner, Ingersoll & Rose, 1996; Diamond, Campbell, Park, Woodson, Conrad, Bachstetter & Mervis, 2006; Ennaceur, Neave & Aggleton, 1997; Luine, 2008) and because BPA alters spine synapse density in these areas (MacLusky et al, 2005; Leranth et al, 2008 a,b). In addition, we measured the activity of several proteins previously described as having an effect on memory consolidation processes, including *N*-methyl-D-aspartate receptors (NMDAR; Morris, 1989; Steele & Morris, 1999), cAMP-responsive element-binding protein transcription factor (CREB; Kida et al. 2002; Han et al. 2009), protein kinase M-zeta (PKM Migues, Hardt, Wu., Gamache, Sacktor, Wang & Nader K, 2010; Pastalkova, Serrano, Pinkhasova, Wallace, Fenton, & Sacktor, 2006; Serrano, Friedman, Kenney, Taubenfeld, Zimmerman, Hanna, Alberini, Kelley, Maren, Rudy, Yin, Sacktor & Fenton,

2008; Sacktor, 2008), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR; Malinow & Malenka, 2002, for review), brain-derived neurotrophic factor (BDNF; Bekinschtein, Cammarota, Izquierdo & Medina, 2008; Binder & Scharfman, 2004, for review), and post-synaptic density-95 (PSD-95; Ehrlich, Klein, Rumpel & Malinow, 2007; Migaud et al., 1998), in order to reveal possible underlying mechanisms of BPA on memory and synaptic plasticity. We hypothesized that acute BPA exposure would impair memory in adult male rats, reduce spine density in the hippocampus and mPFC, and block the activity of memory consolidation related proteins in those areas.

Methods

Subjects

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) aged 60 days upon arrival, were used. Animals were treated according to the NIH Guide for Care and Use of Laboratory Animals, and the Hunter College Institutional Animal Care and Use Committee approved the experiment. Two rats were housed in each cage, and access to food and water was unlimited during the entire period of the experiment. Animals were kept under a 12-hour light/dark cycle, and were allowed to acclimate to the housing environment for a week before starting habituation trails. Body weights were taken every 2-3 days.

Materials and Procedure

Memory Tasks—Memory abilities were assessed using object recognition (OR) and object placement (OP) tasks. These tasks were developed by Ennaceur, Delacour and Meliani (Ennaceur & Delacour, 1988; Ennaceur & Meliani, 1992) and were successfully used over the years as a non-stressful measurements of both visual, non-spatial, PFC-dependent memory (OR), as well as spatial, hippocampal-dependent memory (OP) in rats (Beck & Luine, 2002; Bisagno, Bowman & Luine, 2003; Bowman et al., 2004; Ennaceur, Neave & Aggleton, 1997; Luine, Jacome & Maclusky, 2003; Salas-Ramirez, Frankfurt, Alexander, Luine & Friedman, 2010). Both tasks begin with a sample trial (T1) in which the rat is placed in an open field box with two identical objects, and is allowed to explore the box and objects for three minutes. A total object-exploration time is measured in order to ensure that the rat explore the objects sufficiently. After an inter-trial interval, the rat is returned to the box for a three-minute recognition trial (T2). In the OR task, one of the identical objects is replaced by a different object at T2, while in the OP task, one of the identical objects is placed in a different location at T2. In both tasks, the time the rat spends with the new (novel) object/location versus the time spent with the old (familiar) object/location is measured. This measurement is based on the notion that rats like novelty. If the rat remembers the old object/placement, it will spend more time exploring the new one, however, if the rat does not remember the old object/placement, it will spend similar time with both of them.

To acclimate the animals to the field and to the task, all subjects received an open field trial trial, followed by OR and OP trials, before being tested. The inter-trial delays were 1, 15, 60 and 120 minutes for the OR habituation, and 10, 40, 60 and 120 minutes for the OP habituation. The objects that were used included different kinds of bottles, cans and containers, and a new set of objects was used for every trial during both habituations and tests. During T2, novel objects/placements were counterbalanced across treatment. Exploration was defined as sniffing at, whisking at, or observing the object from within approximately 2 cm.

On the day of testing, subjects received a three minute T1 session and then were immediately injected with saline or BPA (See BPA dosage below). Two hours later, they

received T2 recognition trials. Subjects that did not explore the objects during either T1 or T2 (total exploration time less than 10 seconds), or knocked over the objects, were excluded from the data analysis. Thus, one subject (control) was excluded from OR data analysis and two subjects (control and BPA) were excluded from OP data analysis.

BPA Dosage—10mg of BPA was dissolved in 1ml of ethanol for a stock solution that was stored at −20C°. On the day of the tests, injection solutions were made by diluting 40μl of stock solution in 9.96ml of saline for the BPA solution, and 40μl of ethanol in 9.96ml of saline for the control solution. On the day of each test, cage mates received a subcutaneous (SC) injection of either 40μg/kg of BPA or saline solutions. It is important to note that, in order to prevent a potentially stressful experience on the day of the tests, animals were also habituated to SC saline injections during the behavioral habituations.

Golgi Impregnation—40 minutes after a T1, sample trial, rats were sacrificed by rapid decapitation, and the brains removed and cut in half sagitally. One of the hemispheres (right and left hemispheres were counter-balanced) was further divided into an anterior and posterior block; one containing the mPFC and the other containing the hippocampus, and processed for Golgi impregnation (the other was prepared for Western blotting; see below). Blocks were placed directly in solutions from the FD Rapid GolgiStain Kit (FD Neuro-Technologies, Inc., MD, USA) and kept at room temperature in the dark. Solutions were replaced after 24 hours and blocks were kept for two weeks. Blocks were then placed in solution C (a sucrose-based solution) for another 48 hours and then frozen on dry ice and stored at −70C° until sectioning. Cryostat sections of 100μm were cut at −25C°, mounted on gelatin-coated slides, and air dried in the dark at room-temperature. When dry, sections were rinsed with distilled water, placed in a silver nitrate containing solution for 10 minutes, rinsed again with distilled water, and then dehydrated in 50%, 75%, 95% and 100% ethanol for five minutes each. Finally, sections were cleared with Protocol (Fischer Scientific) three times for five minutes each and cover slipped with Permount (Fischer Scientific).

Dendritic Spine Density Analysis—Using a Leitz Diaplan microscope, a Nikon DXM 1200F camera, and Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA), dendritic spine density on pyramidal cells from the CA1 region of the dorsal hippocampus and layer II/III of the mPFC was analyzed. Regions were defined based on *The Rat Brain Atlas in Stereotaxic Coordinates* (Paxinos and Watson, 1998), and counting was done as described by Frankfurt, Wang, Marmolejo, Bakshi & Friedman (2009). In short, for each subject, spines from six secondary basal dendrites and six tertiary apical dendrites were counted under oil (x100) in each of the brain areas. The dendrites chosen had to be clearly stained, unbroken and isolated from other cells' dendrites in order to allow for accurate counting. Spine density was calculated by dividing the number of spines by the length measured for that dendrite. The average of six dendrites/subject, expressed as spines/10μm dendrites, was obtained for each subject, and then a group average was obtained. See Fig. 2 for photomicrograph of representative dendrites.

Corticosterone Assay—Trunk blood was collected at sacrifice, in order to determine corticosterone (CORT) levels. Blood was allowed to separate at room temperature, centrifuged at 4C° for 15 minutes at 2600g. Plasma was collected and stored in sealed tubes at −80C° until further processing. Following extraction with ether, free CORT levels were measured by ELISA, using the Neogen Corporation Corticosterone kit, according to kit protocol (Neogen Corporation, MI, USA).

Protein Fractionation—In order to isolate proteins from nuclear, cytosolic and synaptic membrane compartments, a subcellular fractionation procedure was performed as described

by Sacktor et al., 1993. In short, immediately after sacrifice and brain removal, hippocampus and PFC from one hemisphere were dissected on dry ice, and stored at −80C° until further processing. Tissue was homogenized in a Tris buffer containing a combination of protease inhibitors, and centrifuged at 4C° for 5 minutes at 3000g. Supernatant (S1) was separated from pellet (P1; nuclear fraction), and ultracentrifuged at 4C° for 30 minutes at 100,000g. The pellet (P2), from this spin was separated from the supernatant (S2; cytosolic fraction) and resuspended in a Tris buffer containing 0.05% Triton-X and set at 4C° for one hour, following by ultracentrifugation at 4C° for 60 minutes at 100,000g. The pellet from this spin (P3; synaptic fraction) was separated from supernatant (S3) and resuspended in Tris buffer. The samples were then diluted with 5X Laemmli sample buffer (BioRad, Hercules, CA) containing β-mercaptoethanol, denatured on a hot plate and stored at −80C° until analysis.

Western Blot Analysis—In both hippocampus and mPFC, levels of NMDA-R2b, AMPA-GluR2, PSD-95 and PKMζ were measured in the synaptic fraction, levels of pCREB, PKMζ and BDNF were measured in the cytosolic fraction, and levels of pCREB were measured in the nuclear fraction. To ensure equivalent amounts of total protein were loaded for all samples, a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) was used in order to determine the concentration of proteins in each of the fractions, and Tubulin was used as loading control to confirm total protein levels. The protein samples were loaded onto 4-20% Tris-glycine gradient gels (Lonza Group Ltd., ME, USA) and run at a constant 200V, 200amp, for 45 minutes, in electrophoresis tanks (C.B.S. Scientific Co., CA, USA) with TBS buffer (Lonza Group Ltd., ME, USA). The proteins, then, were transferred from the gels onto a 0.5μm nitrocellulose membrane at a constant 200V, 100amp, for three hours. The membrane was removed from the gel, stained with Ponceau stain, blocked, and incubated over night in primary antibodies.

The primary antibodies used were diluted in a hemoglobin based blocker at the following concentrations: NMDA-R2b (Abcam Inc., MA, USA) at 1:1000, AMPA-GluR2 (Millipore Co., CA, USA) at 1:1000, PSD-95 (Millipore Co., CA, USA) at 1:1000, PKMζ (Santa Cruz Biotechnology, Inc., CA, USA) at 1:1000, pCREB (Abcam Inc., MA, USA) at 1:250, BDNF (Sigma-Aldrich Co., MO, USA) at 1:100, Tubulin (Calbiochem Inc., CA, USA) at 1:2000. Proteins were detected at the following molecular weights: NMDA-R2b at 148 kD, AMPA-GluR2 at 102 kD, PSD-95 at 95 kD, PKMζ at 55kD, pCREB at 37kD, precursor BDNF at 28kD, mature BDNF at 16 kD, and tubulin at 55kD. Membranes were then washed and incubated in secondary antibodies (1:1000 dilution; Sigma-Aldrich Co., MO, USA) for two hours. Membranes were incubated in BCIP/NBT phosphatase substrate for colorimetric detection (KPL Inc., MD, USA) until staining became sufficiently clear. Membranes were then washed, dried and scanned, and band density was measured using the NIH ImageJ software (Bethesda, MD). All sample values were normalized to the tubulin value for the same sample and expressed as arbitrary units (AU, Sacktor et al, 1993).

Procedure—Two experiments were performed. In the first experiment, rats were given a week of acclimation to the housing, following by two weeks of habituation to the OR and OP memory tasks, as well as to the SC injections. Rats were injected S.C. with saline (n = 7) or BPA (n = 7) immediately after T1, and memory was tested after a two-hour delay (T2). Four days after the OR test, an OP test was given in the same manner. Groups were switched between the tasks, so that control subjects from the OR test got BPA in the OP test, and vice versa. BPA has a half life of about six hours (Long et al., 2000) so lingering effects would not be present. Eleven days after the last test, subjects were given T1 once more, followed by an immediate SC injection (assignment to groups was similar to that of OR), and were sacrificed by rapid decapitation 40 minutes after injection. Brains were then removed, dissected and taken for morphological and chemical analysis. Trunk blood samples were taken in order to measure corticosterone levels.

In the second experiment, the effects of BPA by itself (without memory consolidation) on dendritic spine density were examined. After acclimation and habituation to SC injections, rats were injected with saline (n = 7) or BPA (n = 7) subcutaneously, without an object exploration trial (T1). Rats were sacrificed 4 hours post-injection, and brains were prepared for Golgi staining and dendritic spine analysis as described above. The two Golgi experiments were processed and analyzed separately because we have repeatedly demonstrated that there is little variability between experiments (Luine and Frankfurt, 2011).

Data Analysis—All data were analyzed using SPSS software (Systat Inc., Chicago, IL., USA). The behavioral data were analyzed using a two-way repeated measures ANOVA (Group x Object) and differences tested using a post hoc one-tailed, paired *t*-test between the time spent with the new vs. old objects/placements during T2 in each of the groups (BPA and control). Apical and basal spine densities were analyzed by two-way ANOVA, Group (Control, BPA) x Area (CA1, mPFC), and differences tested, where appropriate, by post hoc t-tests. Differences in corticosterone levels (Control vs. BPA) were assessed by two-tailed *t*-test. Differences between Control and BPA for the protein analyses were determined by the non-parametric, Mann-Whitney U-test because the gel density data, when transformed, were not normally distributed and therefore did not conform to parametric statistical analyses. All differences were considered significant at the $p < 0.05$ level.

Results

Behavior

In the object recognition (OR) task, a two-way repeated measures ANOVA showed a significant effect of group ($F1,22 = 9.92$, $p < 0.05$) and object ($F1,22 = 6.03$, $p < 0.02$) but no interaction effect ($F1,22 = 2.83$, $p = 0.11$). Post hoc t-tests showed that animals in the control group significantly discriminated between the old and new objects ($t = 3.232$, $p < 0.05$), while animals administered BPA did not, spending similar time exploring both old and new objects ($t = 0.645$, $p > 0.05$, Fig. 1A). In the object placement task (OP), similar results were obtained: Two way ANOVA showed a significant group ($F1,20 = 4.85$, $p < 0.04$) and object location ($F1,20 = 7.08$. $p < 0.02$) effect but no interaction effect ($F1, 20 = 0.80$, $p = 0.38$). Controls significantly discriminated between the old and new placements ($t = 3.045$, $p < 0.05$), while animals administered BPA did not ($t = 1.528$, $p > 0.05$, Fig. 1B). Taken together, these results suggest that acute administration of 40μg/kg BPA impairs both visual and spatial memory in adult male rats.

Morphology

Rats were sacrificed 40 min following a T1 trial, and dendritic spine density in the CA1 sub region of the hippocampus and layer II/III of the mPFC was measured. Apical and basal spine densities were analyzed separately by two-way ANOVA, Group (Control, BPA) x Area (CA1, mPFC), and differences tested, where appropriate, by post hoc t-tests. For Apical spines, there was a significant group ($F1, 24 = 17.0$, $p < 0.0001$) and area ($F1, 24 = 8.51$, $p < 0.008$) effect but no significant interaction effect ($F1, 24 = 3.99$, $p < 0.057$). For basal spines, there was a significant group ($F1, 24 = 13.16$, $p < 0.001$) effect but no significant area ($F1, 24 = 2.54$, $p < 0.12$) or interaction effect ($F1, 24 = 3.54$, $p < 0.072$). Post hoc *t*-tests revealed significantly lower spine densities in the BPA groups compared with controls (Fig. 2 and Fig. 3A). This effect was found on both the apical (10% decrease; $t = 3.547$, $p < 0.01$) and basal (9% decrease; $t = 2.248$, $p < 0.05$) dendrites of pyramidal cells in the hippocampus, as well as apical (23% decrease; $t = 3.208$, $p < 0.01$) and basal (26% decrease; $t = 2.989$, $p < 0.05$) dendrites of pyramidal cells in the mPFC. Spine density values were higher than previously reported (Frankfurt, Salas-Ramirez, Friedman & Luine, 2011; Frankfurt et al., 2009; Fig. 3A); therefore we measured spine density in an additional group

of subjects that received the same dose of BPA but did not undergo T1 memory tests. No significant difference in dendritic spine density was found between the groups in either the CA1 or mPFC, and all values were generally lower than in rats that underwent T1 memory trials (Fig. 3B).

Together, these results suggest that high dendritic spine density in the CA1 and mPFC is associated with memory consolidation processes, and that in the absence of such processes, this increase in spine density is moderated. Moreover, acute administration of 40μg/kg BPA blocked the formation of new spines both in the hippocampus and the mPFC during memory consolidation, a change that may be linked to the memory impairments observed in animals treated with BPA.

Hormones

There was no significant difference between the groups in their corticosterone levels (t = 0.201, $p > 0.05$). This result suggests that acute administration of 40μg/kg BPA does not affect corticosterone levels, and that the observed memory impairment was not due to stress induced by BPA.

Biochemistry

In the hippocampus, controls had higher levels of PSD-95 as compared to the BPA group ($U = 8$, $p < 0.04$; Fig. 4). In the mPFC, BPA treatment led to higher levels of pCREB in the cytosolic fraction ($U = 5$, $p < 0.05$), and lower levels of pCREB in the nuclear fraction, compared to controls, although nuclear results did not reach statistical significance (nucleus: $U = 7$, $p = 0.051$; Fig. 5). In addition, Figures 4 and 5 show no changes in NMDAR-2b, AMPAR-GluR2, PKMζ and pre-BDNF in either brain area of BPA treated subjects. These results suggest that the observed memory impairment and reduction in synaptic plasticity followed by acute administration of BPA, might be mediated by changes in PSD-95 and pCREB pathways.

Discussion

The results of the current experiments suggest that acute BPA exposure at a dose below the U.S.E.P.A. reference safe daily limit (U.S.E.P.A., 1993) antagonizes memory consolidation in adult male rats. BPA impaired memory in both the OR and OP tasks, and was associated with lower densities of dendritic spines, from approximately 10-25%, in both CA1 and the mPFC. This is the first report showing that acute BPA administration in adult males induces memory deficits and is associated with reduced dendritic spine density. These results are in agreement with, and add to, previous studies that demonstrated BPA-induced memory impairment during development (Carr et al., 2003; Goncalves et al., 2010; Xu et al., 2010) and adulthood (Inagaki et al., 2007), and a blocking effect of BPA on estradiol and testosterone dependent formation of dendritic spine synapses in male and female rodents (MacLusky et al., 2005; Leranth et al., 2008a, 2008b). We also identified a decrease in PSD-95 in the post synaptic density in the hippocampus and an increase in cytosolic pCREB in the mPFC in BPA treated males.

In this study we examined BPA effects on memory consolidation, a process that is believed to take place in the first two hours after acquisition of new knowledge and involves many cellular signaling pathways that result in synaptic changes. The association between memory and spine synapses has been supported in several studies (Leuner, Falduto & Shors, 2003; Luine, Attalla, Mohan, Costa & Frankfurt, 2006; Wallace, Luine, Arellanos & Frankfurt 2006; Luine & Frankfurt, 2011); however, the specific role of these morphological changes in memory consolidation processes is less clear. It has been shown that both estrogens and

androgens can facilitate a rapid enhancement in synaptic density, in both the CA1 region of the hippocampus and mPFC in rodents (Hajszan, MacLusky, Johansen, Jordan & Leranth, 2007; Leranth, Petnehazy & MacLusky, 2003; Leranth, Hajszan & MacLusky, 2004; MacLusky, Luine, Hajszan & Leranth, 2005; Inagaki et al., 2007), as well as in non-human primates (Hao et al., 2003, 2006; Leranth, Shanabrough & Redmond 2002). Moreover, it has been demonstrated that immediate post-training treatment with estrogens, but not a delayed treatment (1-4h post-training), can enhance memory across different tasks (Luine et al., 2003; McGaugh, 2000; Packard and Theather 1997a,b; Rhodes and Frye, 2004). Fewer studies have assessed gonadal hormone influences on male memory, but castration is associated with declines in spatial working memory, and both estrogens and androgens can restore performance in some, but not all, memory tasks (Luine, 2008). Taken together, these studies strongly suggest that gonadal hormones are involved in memory consolidation processes by increasing synaptic density in CA1 and mPFC. In relation to BPA effects on spine synapses, a previous study showed that 300 μg/kg of BPA, given for 4 days, reduced spine synapses in the PFC and CA1 area of adult male rats by approximately 50% (Leranth et al, 2008b). Moreover, MacLusky et al (2005), using a dose and time course of BPA treatment which is more similar to our current study (40 ug/kg of BPA given at the same time as 60 ug/kg of estradiol), showed a significant antagonism in estrogen-dependent spine synapse formation 30 min later in OVX female rats. Our study provides further understanding of the role of synaptic plasticity in memory consolidation processes. When memory consolidation was involved (experiment 1), dendritic spine density values were higher than when memory was not involved (experiment 2). These values were also higher than previously reported in morphological studies from our lab (Frankfurt et al., 2009; Frankfurt et al., 2011; Luine and Frankfurt, 2011) in which memory consolidation was not involved. Thus, our study demonstrates, for the first time, that memory consolidation is consistent with higher dendritic spine density in both CA1 and mPFC, and that BPA can interfere with this increase. However, it is not clear whether the current behavioral and morphological changes involve action of BPA on estrogen or androgen receptors since BPA can antagonize hormone actions at both receptors (See Leranth et al, 2008b; Wolstenholme, et al, 2011 for further discussion).

Another goal of this study was to elucidate possible mechanisms underlying the effects of BPA on dendritic spine density in the hippocampus and mPFC that may alter memory processes. Poimenova, Markaki, Rahiotis and Kitraki (2010) demonstrated that BPA exposure during development led to increased corticosterone levels in mid-adolescent male rats following performance of the Y-maze task and impaired memory. One possible explanation for our results, therefore, is that the subjects had increased corticosterone levels following BPA exposure and memory task conditions, and therefore performed poorly at the memory challenge (T2). Indeed, several studies have shown that acute stress, and the resulting elevation in corticosterone levels, can impair memory in young adult male rats (Diamond et al, 1996; Diamond, Park, Heman & Rose, 1999; Diamond et al., 2006; Woodson, Macintosh, Fleshner & Diamond 2003; Sandi et al., 2005; Park et al., 2006; Park, Zoladz, Conrad, Fleshner & Diamond, 2008), and block enhancement of dendritic spine density in the hippocampus (Diamond et al., 2006). However, measurement of corticosterone levels in our study revealed no significant difference between the groups, ruling out the possibility that BPA impaired memory indirectly by elevating the levels of this stress-hormone.

A remaining caveat of the study is that BPA treatment led to increased exploration during T2 in the object recognition test and decreased exploration during T2 in the object placement test (Figure 1). There were, however, no group differences in exploration during T1 which might influence performance in T2 (data not shown). It should be noted that BPA treatment was alternated among the subjects so that BPA treated subjects in the object

recognition test were the controls for the object placement test. Thus, the exploration difference appears to be due to more exploration in half of the cohort and not to a BPA effect on exploration. In support, preliminary experiments for this study, conducted in other subjects, did not show effects of BPA on exploration in either T1 or T2 (Eilam-Stock and Luine, unpublished observation).

The rapid effect of gonadal hormones on memory and neural plasticity strongly suggest that these effects involve non-genomic mechanisms. Recent research revealed many possible pathways by which gonadal hormones might affects these processes, including interactions with pCREB (Abraham & Herbison, 2005; Szego et al., 2006; Wade & Dorsa, 2003), BDNF (Scharfman & MacLusky, 2006; Solum & Handa, 2002), NMDAR-2b (Adams, Fink, Janssen, Shah & Morrison, 2004; d'Anglemont de Tassigny et al., 2007) and PSD-95 (Akama & McEwen, 2003; d'Anglemont de Tassigny et al., 2007; Li et al., 2004). Since BPA is an endocrine disruptor with anti-estrogen/androgen properties, it might impair memory and synaptic plasticity processes by blocking proteins that are activated in cell-signaling pathways and memory consolidation. Data regarding the involvement of BPA in such mechanisms are very limited, though some studies suggest that BPA effects pCREB (Canesi, Betti, Lorusso, Ciacci & Gallo, 2005; Quesada et al., 2002), and NMDA receptor subunit NR2B (Xu et al., 2010a, b). In our experiment, we examined the levels of these two proteins, in addition to other memory process related proteins, in the hippocampus and the mPFC. No significant differences between groups in the levels of NMDA-R2B, BDNF, PKMζ, and AMPA-GluR2, in either the hippocampus or the PFC were found in the present study. However, significantly higher levels of PSD-95 in the post synaptic density fraction of the hippocampus of control subjects were found as compared to BPA-treated subjects. This finding is consistent with the BPA-dependent decrease in spine density and relevant to the memory impairments by BPA, since PSD-95 is believed to play an important role in synaptic plasticity by facilitating the binding of receptors and channels to the post-synaptic membrane (see Kim & Sheng, 2004; van Zundert, Yoshii & Constantine-Paton, 2004, for review). In addition, we found lower nuclear (not statistically significant) and higher cytosolic levels of pCREB in the PFC of BPA treated subjects. Since CREB is a transcription factor that is active in the cell's nucleus when phosphorylated, it might be hypothesized that BPA is preventing translocation of pCREB to the nucleus and may also be secondarily altering transcription factor functions. The absence of significant effects on nuclear pCREB and other memory consolidation related proteins in this study, may be related to the specific time of sacrifice (40 minutes post injections and sample trials, T1, of the memory task). It is possible that some of these proteins are activated earlier or later in the memory consolidation process and that other BPA effects would be observed at other sacrifice times. Future studies employing a more detailed time course following the sample trial are needed in order to further clarify the underlying mechanisms of BPA's actions.

The results of the present study suggest a relationship between memory performance and dendritic spine density in the hippocampus and mPFC. However, since animals were sacrificed 11 days after the last memory test, neuroanalysis cannot be directly correlated to memory performance. Even though we found altered neuronal processes after BPA exposure in animals that displayed BPA-induced memory impairment a few days prior to sacrifice, we cannot determine whether BPA actually blocked the formation of a new memory on the day of sacrifice. The rational for this design was our hypothesis that BPA interrupts memory consolidation processes rather than memory retrieval. Additionally, it is important to note that on the day of sacrifice, all subjects explored the objects for well over the 10 second threshold, making it likely that the animals indeed formed a new memory. This issue requires further investigation in order to determine whether the observed memory impairment was due to BPA interference with memory consolidation processes or memory retrieval.

Since BPA was administered subcutaneously, rather than orally, in the present study, one might argue that our results are not relevant to the actual risks of human exposure to BPA. In a preliminary study, however, Hajszan & Leranth (2010) reported no differences between SC and oral administration of BPA on the percent of reduction in spine synapses in both the hippocampus and the PFC, and Taylor, Welshons & VomSaal (2008) found no differences in circulating BPA following oral or SC dosing in neonatal mice. Therefore, SC injections seem to be viable and relevant for modeling potential effects of BPA in humans.

In conclusion, our findings demonstrate, for the first time, that BPA can impair memory in adult male rats and block synaptic plasticity processes. Moreover, our results suggest novel evidence for the underlying biomolecular mechanisms of BPA's actions in memory-related areas of the brain. These results have significant clinical implications, since the BPA dose used in our experiments was 20% lower than the U.S. E.P.A. recommended safe daily limit (50 ug/kg/day). BPA is produced at an estimated rate of 6.5 billion pounds a year, and people are exposed to it on a daily basis. In Canada and the European Union, BPA has been eliminated from use in baby products, where its effects are most profound. In the United States, however, no such steps have been taken to reduce human exposure to BPA, and the status of BPA as an environmental hazard is still under debate. Our study affords a better understanding of the effects BPA exerts on memory, neural plasticity and their biochemical mechanisms, as well as its level of potential danger to society at large.

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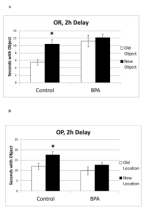


Figure 1. Effects of BPA on recognition memory tasks

A. Object Recognition. The time spent exploring the old (□) and new (■) objects during retention trial (T2), is shown for control (n = 6) and BPA-treated (n = 7) subjects. Vehicle or BPA (40μg/kg) was given immediately after sample trial (T1), and inter-trial delay was 2h. Entries are the average ± SEM. Data analyzed by two-way ANOVA (see text), and differences were tested in each group by paired *t*-test, $*p < 0.05$. **B**. Object Placement. The time spent exploring the old (□) and new (■) locations during retention trial (T2), is shown for control (n = 6) and BPA-treated (n = 6) subjects. Vehicle or BPA (40μg/kg) was given immediately after sample trial (T1), and inter-trial delay was 2h. Entries are the average ± SEM. Data analyzed by two-way ANOVA (see text), and differences were tested in each group by paired *t*-test, $*p < 0.05$.

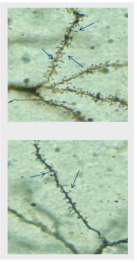


Figure 2. Photomicrograph from the mPFC

Representative photomicrographs illustrating the effect of BPA on dendritic spine density on pyramidal cells of Layer II/III in the mPFC. Arrows denote spines on secondary basal dendrites in vehicle (upper picture) and BPA (bottom picture) treated subjects. 100x under oil.

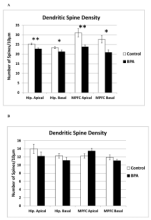
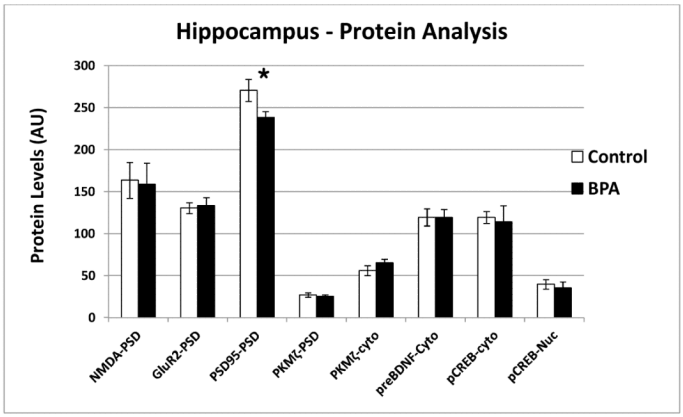


Figure 3. Effects of BPA on dendritic spine density of pyramidal cells in CA1 and mPFC

Entries are the average number of spines/10μm ± SEM. **A.** Experiment 1. All subjects received a sample trial (T1) and an immediate post-trial injection of either vehicle (n = 7) (□) or BPA (40μg/kg) (n = 7) (■). Animals were sacrificed 40 minutes after injections, and apical and basal spine densities were analyzed separately by two-way ANOVA, Group (Control, BPA) x Area (CA1, mPFC), and differences tested, where appropriate, by post hoc t-tests (see results section for ANOVA outcomes). *t*-test, **p* < 0.05, ***p* < 0.01. **B.** Experiment 2. No sample trial was given. Subjects were injected with vehicle (n = 7) (□) or BPA (40μg/kg) (n = 7) (■), and were sacrificed 4 hours after injections. Apical and basal spine densities were analyzed separately by two-way ANOVA, Group (Control, BPA) x Area (CA1, mPFC). No significant main or interaction effects for either apical or basal spines were found.

A



B

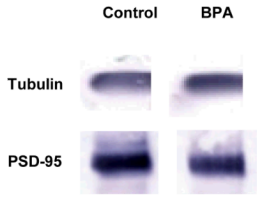


Figure 4. BPA effects on protein levels in the hippocampus

A. Protein levels in each sample were measured by NIH Image-J program, normalized for tubulin concentration and expressed in Arbitrary Units (AU). Entries are the average ± SEM for control (n = 7) (□) and BPA-treated (n = 7) (■) subjects. Differences between the groups were tested by Mann-Whitney U-test, *$p < 0.04$. **B.** Representative immunoblots of Tubulin (loading control) and PSD-95 in the cytosolic fraction of hippocampus.

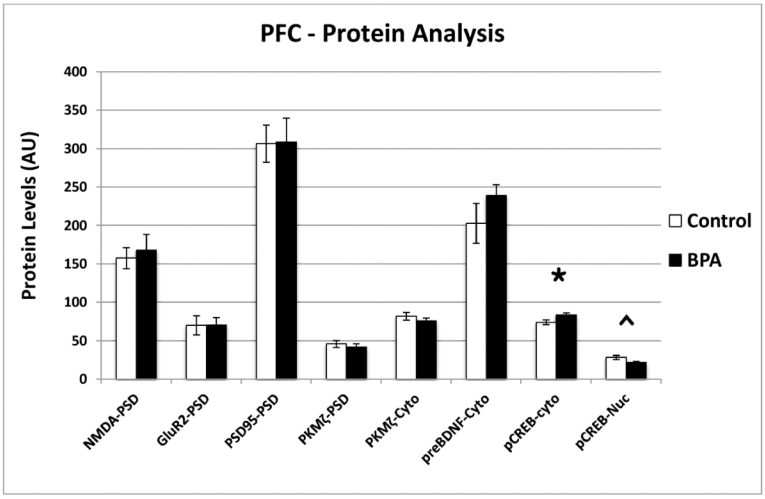


Figure 5. BPA effects on protein levels in the PFC

Protein levels in each sample were measured by NIH Image-J program, normalized for tubulin concentration and expressed in Arbitrary Units (AU). Entries are the average ± SEM for control (n = 7) (□) and BPA-treated (n = 7) (■) subjects. Differences between the groups were tested by Mann-Whitney U-test, * $p < 0.05$, ^ $p = 0.051$.