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Adolescent Bisphenol-A exposure decreases dendritic spine density: Role of sex and age

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Abstract

Bisphenol-A (BPA), a common environmental endocrine disruptor, modulates estrogenic, androgenic, and anti-androgenic effects throughout the lifespan. We recently showed that low dose BPA exposure during adolescence increases anxiety and impairs spatial memory independent of sex. In the current study, six week old Sprague Dawley rats (n=24 males, n=24 females) received daily subcutaneous injections (40 μg/kg bodyweight) of BPA or vehicle for one week. Serum corticosterone levels in response to a 1 h restraint stress and spine density were examined at age 7 (cohort 1) and 11 (cohort 2) weeks. Adolescent BPA exposure did not alter stress dependent corticosterone responses but decreased spine density on apical and basal dendrites of pyramidal cells in the medial prefrontal cortex (mPFC) and hippocampal CA1 region (CA1). Sex differences in spine density were observed on basal dendrites of the mPFC and CA1 with females having greater spine density than males. This sex difference was further augmented by both age and treatment, with results indicating that BPA-dependent decreases in spine density were more pronounced in males than females on mPFC basal dendrites. Importantly, the robust neuronal alterations were observed in animals exposed to BPA levels below the current U.S.E.P.A. recommended safe daily limit. These results are the first demonstrating that BPA given during adolescence leads to enduring effects on neural morphology at adulthood. Given that humans are routinely exposed to low levels of BPA through a variety of sources, the decreased spine density reported in both male and female rats after BPA exposure warrants further investigation.

Keywords

hippocampus; medial prefrontal cortex; corticosterone

Bisphenol-A (BPA), a known endocrine disruptor, is utilized in the manufacturing of hard plastic products such bathtubs, countertops, and microwaveable food containers. Potential

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health hazards exist because alterations in temperature and pH can cause leaching of BPA from plastics (Rubin, 2011; Rubin and Soto, 2009; Talsness et al., 2009; vom Saal and Hughes, 2005) and detectable levels of BPA have been reported in saliva, urine, blood, breast milk and the placenta of humans and animals (Biedermann et al., 2010; Geens et al., 2011; Rubin, 2011). Exposure to BPA has been documented to have estrogenic, anti-estrogenic, and anti-androgenic effects (Negishi et al., 2003; Sohoni and Sumpter, 1998) on various hormone- induced physiological and behavioral phenomena. Perinatal exposure to BPA has been shown to reverse and abolish sexual dimorphisms in several neural regions. For example, perinatal BPA exposure decreased the number of tyrosine hydroxylase cells in the anteroventral periventricular nucleus of the hypothalamus (Patisaul et al., 2006) and the rostral periventricular preoptic area (Rubin et al., 2006) in female rats. In rhesus monkeys, prenatal exposure to BPA decreased fetal midbrain tyrosine hydroxylase neurons and reduced the number of spine synapses in the CA1 region of the hippocampus (Elsworth et al., 2013). Sex differences in overall volume of the locus coeruleus are abolished by BPA in exposed female rodents (Kubo et al., 2003). Additionally, perinatal BPA exposure reverses the sexually dimorphic patterns of estrogen receptor beta mRNA expression in the hypothalamus and amygdala of rats (Cao et al., 2013).

Perinatal BPA exposure also alters a wide variety of behaviors. Prenatal BPA exposure led to the abolishment of sex differences in open-field behavior (Fujimoto et al., 2006; Kubo et al., 2003) and forced swimming test (Fujimoto et al., 2006) in adolescent rats. Chronic low dose perinatal exposure to BPA increased aggression and anxiety in adult rats (Patisaul and Bateman, 2008; Patisaul et al., 2012) and decreased exploratory behaviors in both adolescent (Fujimoto et al., 2006) and adult rodents (Farabollini et al., 1999; Goncalves et al., 2010). Acute administration of BPA during the perinatal period has been observed to increase hyperactivity in adolescent males in a dose dependent fashion (Ishido et al., 2004; Kiguchi et al., 2008). Postnatal BPA exposure (i.e., during lactation) impaired both object recognition and spatial memory in male and female adult rats (Goncalves et al., 2010). Taken together these experiments demonstrate that early BPA administration has behavioral effects on a variety of non-reproductive behaviors.

The mechanism (s) by which BPA exerts its effects on neural systems appears to be as a mixed agonist/antagonist for both estrogen and androgen receptors (Wolstenholme et al., 2011). BPA can, depending on the dose and the presence or absence of circulating gonadal hormones, mimic or block gonadal hormone effects on neural functions such as memory. When an acute dose, 40 μg/kg, of BPA is given to intact adult males immediately following a sampling trial, object and place memory is impaired 2–4 hours later (Eilam-Stock et al., 2012). In cycling rats, the impairment of object memory by BPA occurred during proestrous (Inagaki et al., 2012). Therefore, BPA rapidly impairs hormone dependent recognition memory in gonadally intact adult male and female rats. However, BPA does not appear to mimic estradiol's effects on memory in females because doses from 1 to 400 μg did not affect recognition memory in ovariectomized (OVX) females, but 40 μg/kg BPA blocked recognition memory enhancements in OVX females receiving 17β-estradiol (Inagaki et al., 2012). Thus, when administered with estrogen, BPA appears to rapidly antagonize estrogenic / androgenic effects on recognition memory, but alone it does not enhance or impair memory.

There is increasing evidence that the mechanism(s) underlying estrogenic effects on learning and memory involve plasticity of dendritic spines on pyramidal cells in the CA1 region of the hippocampus (CA1) and the medial prefrontal cortex (mPFC) (for review, Luine and Frankfurt, 2012). Although early studies demonstrated that chronic changes in estradiol altered dendritic spine density, more recently it has been demonstrated that acute administration of estrogen also rapidly increases dendritic spine density and enhances memory (Luine et al., 2003; MacLusky et al., 2005a). In addition, acute administration of BPA alters gonadal hormone effects on both memory and dendritic spines. In males, within 30 to 40 minutes BPA (40 μg/kg) impairs recognition memory and decreases spine density in the apical and basal dendrites of the mPFC by approximately 25% and CA1 by approximately 10% (Eilam-Stock et al., 2012). In females, BPA also blocks estrogen dependent enhancements in recognition memory, but BPA interactions with estrogen on spines appears more complex than in males. Given 30 min before estradiol (20 μg/kg), 40 μg/kg of BPA blocks estrogen's induction of spines on basal dendrites in CA1 but is additive with estrogenic effects on basal dendrites in the mPFC at 4 h (Inagaki et al., 2012).

The age of exposure (the vast majority of studies have focused on pre- and neo-natal exposure) and the dose (most focus on chronic, high dose amounts) are important considerations when reviewing the BPA literature. The findings reported above (Eilam-Stock et al., 2012; Inagaki et al, 2012) on BPA's effect on memory and spine density have been demonstrated in adulthood following an acute dosing regimen, but it is currently unclear whether the same pattern of results hold true following adolescent BPA exposure. Adolescence is an important developmental period characterized by hormonal changes which induce structural effects on the brain and subsequently behavior (for review, Romeo, 2003); however, only recently have researchers begun to turn their attention to the behavioral effects of BPA exposure during adolescence (e.g., Diaz-Weinstein et al., 2013; Xu et al., 2011). We have previously demonstrated that short term, low-dose BPA exposure (below the current reference safe daily limit of 50 μg/kg day set by the United States Environmental Protection Agency, 1993) during adolescence increased anxiety on the elevated plus maze and open field and impaired spatial memory on the object placement task, independent of sex (Diaz-Weinstein et al., 2013). To date, there is no literature regarding the potential effects of adolescent BPA exposure on neuronal morphology in brain areas known to contribute to these cognitive behaviors such as the CA1 and mPFC (Broadbent et al., 2004; Jo et al., 2007).

In addition to BPA's effect on sexual differentiation of the brain and numerous behavioral responses, perinatal exposure to low-dose BPA alters corticosterone levels under both basal and stress conditions in adolescence (Panagiotidou et al., 2014; Poimenova et al., 2010). These findings show that perinatal exposure to BPA alters the concentration 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). To date, there is no literature regarding the potential effects of adolescent BPA exposure on stress induced corticosterone levels and it is unknown whether BPA exposure during adolescence can alter sexually differentiated corticosterone release in response to a stressor in adulthood.

In order to better understand the mechanisms responsible for BPA's effects on the adolescent brain, the present study was designed to determine whether or not BPA exposure during adolescence (postnatal days [PND] 42–49) alters spine density in the mPFC and CA1 region of the hippocampus in male and female rats immediately following BPA injections on day 49 (7 weeks of age), and later in adulthood, at 11 weeks of age. In addition, because perinatal exposure to low-dose BPA alters corticosterone levels under both basal and stress conditions in adolescence, we examined whether adolescent BPA exposure altered serum corticosterone levels in response to a restraint stress challenge at 7 and 11 weeks of age, immediately prior to sacrifice.

Materials and Methods

Subjects

Forty-eight experimentally naïve 5 wk old Sprague Dawley rats (n=24 males, n=24 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 and water (Glass water bottles, Ancare Corporation, Bellmore, NY). All animals were weighed regularly. Table 1 illustrates the methodological 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, 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.

Stress challenge

Immediately following injections on the seventh day, half of the subjects (n=6/group) now aged 7 weeks, were exposed to a 1 h restraint stress challenge. The remaining subjects (n=6/group) were allowed to mature to 11 weeks of age and were then exposed to the 1 h restraint stress challenge. Stress was applied 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 h and were then immediately removed and sacrificed via rapid decapitation (between 12:00 – 1:00 pm).

Golgi Impregnation

Following sacrifice, 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 in 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 5 or 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 sacrifice, trunk blood was collected from all subjects. Samples were centrifuged at 3000g at 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). Three-way (treatment X sex X age) ANOVAs were used to test for group differences in spine density and corticosterone levels. Type I error rate was set at 0.05 and Fisher's LSD Tests were used for post-hoc analysis, where appropriate.

Results

Medial Prefrontal Cortex (mPFC) basilar dendritic spine density

Spine density of basal dendrites in pyramidal cells in the mPFC was measured in control and BPA-treated, male and female rats at 7 and 11 weeks of age, and data analyzed by three-way ANOVA. Figure 1 is of secondary basal dendrites in the mPFC illustrating spine density in a control and BPA treated male. There was no overall treatment X sex X age three-way interaction, $F(1,46)=2.23$, $p=0.14$. Main effects are shown in Figure 2 A. Basal spine density was decreased 21% in all BPA treated subjects (9.4 ± 0.2) compared to all controls (11.9 ± 0.3), regardless of sex or age, main effect of treatment, $F(1,46)=147.82$, $p=0.00001$. There

was a small, 6%, but highly significant sex difference (F (1,46)=8.32, p=0.006), with males, control and BPA treated, having fewer spines on the basal tree of the mPFC neurons (10.4 ± 0.4) than control and BPA treated females (11.0 ± 0.3). The main effect of age showed that all subjects showed a significant 9% increase in spines with time (10.2 ± 0.2 at 7 weeks and 11.1 ± 0.4 at 11 weeks) , F (1,46)=22.38, p=0.0001).

Each of these three main effects were differentiated by three significant two-way interactions. In addition to the main effect of BPA, there was a significant treatment X sex interaction, F(1,46)=9.46, p=0.006. The BPA-dependent decrease in males was 25% while in females it was 15% and post hoc analysis showed that male BPA treated subjects had significant decreased spine density compared to all other groups (Fisher LSD, P=0.05), Figure 2B. Additionally, spine density on basal dendrites of mPFC pyramidal cells showed a significant treatment X age interaction, F (1,46), =55.40, p=0.0001, Figure 2C. Post hoc testing revealed that the combined male and female control subjects had increased spine density from 7 to 11 weeks (Fisher LSD, P=0.05); however, the combined treated subjects did not show increased spine density over time. Thus, the decreased spine density caused by BPA exposure persisted across time in both males and females. Finally, the increase in spine density with age was primarily dependent on changes in males because there was a significant sex X age interaction, F (1,46)=7.70, p=0.008), Figure 2D, such that only males, not females, showed significant increases in spine density from 7 to 11 weeks (Fisher LSD, P=0.05).

Medial Prefrontal Cortex (mPFC) apical dendritic spine density

There was no overall treatment X sex X age three-way interaction effect on apical dendrites of pyramidal cells in the mPFC, F(1,46)=0.18, p=0.67. Main effects are shown in Figure 3A. BPA exposure lead to a 10% decrease in mPFC apical dendritic spine density in the males and females combined (10.1 ± 0.1) compared to the combined controls (11.2 ± 0.2), main effect of treatment, F (1,46)=39.85, p=0.0001. While there was no significant main effect of sex, F(1,46)=1.27, p=0.27, all subjects showed a 9.5% increase in spine density from 7 (10.4 ± 0.2) to 11 weeks (11.5 ± 0.3), main effect of age F (1,46)=16.13, p=0.0003). Furthermore, a significant treatment X age interaction was observed, F (1,46)=10.41, p=0.003 and shown in Figure 3B. Post hoc testing again showed that control subjects increased spine density from 7 to 11 weeks (Fisher LSD, P=0.05); however, BPA exposed subjects had decreased spine density that persisted from 7 to 11 weeks.

CA1 basilar dendritic spine density

There was no overall treatment X sex X age three-way interaction effect in the spine density of basal dendrites in CA1 F(1,44)=0.06, p=0.81. Main effects are shown in Figure 3A. A substantial decrease of 24% was observed in the spine density on basal dendrites in CA1 of all BPA treated subjects (9.1 ± 0.2) compared to all controls (12.0 ± 0.2), main effect of treatment, F(1,44)=103.13, p=0.00001. Figure 4A also shows a smaller, 9%, yet strongly significant sex difference with males, control and BPA treated, having fewer spines on CA1 basal dendrites (9.9 ± 0.4) than control and BPA treated females (10.9 ± 0.3), main effect of sex, F (1,44)=9.19, p=0.004). While there was no main effect of age, F(1,44)=0.90, p=0.35, a significant treatment X age interaction was observed, F (1,44)=6.88, p=0.01 (Figure 4B).

Post hoc testing showed that male and female control subjects had no change in spine density from 7 to 11 weeks; however, both male and female BPA exposed subjects had a significant decrease in CA1 basal dendritic spine density from 7 to 11 weeks (Fisher LSD, P=0.05).

CA1 apical dendritic spine density

The fewest changes in dendritic spine density were observed in the apical dendritic spines of pyramidal cells in CA1. There was no overall treatment X sex X age three-way interaction effect in the spine density of apical dendrites in CA1 $F(1,44)=0.06$, $p=0.81$. Exposure to BPA, regardless of sex or age, significantly decreased spine density by 15% (10.9 ± 0.2) compared to controls (12.9 ± 0.3), main effect of treatment, $F(1,44)=23.06$, $p=0.0002$). No other, significant main or interaction effects were observed in CA1 apical dendritic spine density ($p>.05$).

Serum Corticosterone

All subjects received a 1 h restraint stress challenge at 7 and 11 wks of age, and serum corticosterone levels were measured (Figure 5). There was no overall three-way treatment X sex X age effect on serum corticosterone levels, $F(1,40) = 0.021$, $p=0.89$. While there were no main effects of either BPA treatment or sex ($p>0.05$), corticosterone levels were higher at 11 wks (367.1 ± 42.5, ng/ml) than at 7 wks of age (130.1 ± 30.6, ng/ml), main effect of age, $F(1,47)=23.91$, $p=0.00001$. Additionally, there was a sex X age interaction, $F(1,47)=12.81$, $p=0.0009$. As shown in Figure 5, post hoc testing showed corticosterone levels were lowest in females at 7 weeks compared to all other groups. Furthermore, females had substantial increases in corticosterone levels from 7 to 11 wks; however, males did not have any significant change in levels across time.

Discussion

In the current study, we examined the effects of low dose adolescent BPA exposure on the spine density of apical and basal dendrites of pyramidal cells in the mPFC and CA1 region of the hippocampus, at 7 and 11 weeks of age. These results are the first to demonstrate that short term, low dose exposure to BPA during the critical period of adolescence (PND 42–49) decreases spine density and that, in some cases, these treatment effects are dependent on sex and age of the animal.

Adolescent BPA exposure resulted in a decrease in basilar and apical dendritic spine density in both the mPFC and CA1. In the mPFC, BPA induced decreases in spine density observed at 7 weeks were stable and persisted through 11 weeks. During this time, mPFC apical and basal dendritic spine density in controls increased whereas there were no observed increases in BPA treated subjects across time. These findings differ from recent data demonstrating that dendritic spines decreased in both sexes post-puberty (Koss et al., 2014); however, this apparent discrepancy may be the result of strain differences (hooded versus Sprague Dawley rats). Also, the decreases reported by Koss and colleagues were observed in PFC layer V between PND 35–90 compared to layer II-III between PND 49–77 in the current study. Thus, the observed dendritic pruning may be occurring in the PND 77–90 range.

In CA1, BPA further decreased spine density on basal dendrites with developmental maturation. Control subjects showed no change in basal dendritic CA1 spine density from 7 to 11 weeks of age; however, the BPA decrease observed at 7 weeks persisted through 11 weeks of age. It has previously been shown that acute BPA administration to adult males decreased spine density in both the mPFC and CA1 (Eilam-Stock et al., 2012). Long-term (i.e., 12 weeks) exposure to BPA in adulthood impaired male spatial memory and reduced CA1 hippocampal synaptic density in male mice (Xu et al., 2013). Other evidence has shown that BPA can block the formation of synapses and reduce synaptic density in the CA1 and mPFC (Leranth et al., 2008a,b; McLusky et al., 2005b) in adults. The current data provides novel results that generalize these findings to adolescence and extends them to females. While it is not clear how BPA decreases spine density, changes in cell-signaling pathways (e.g., pCREB, Eliam-Stock et al., 2012) and decreases in NMDA subunit NR1 and AMPA receptor subunit GluR1 binding (Tian et al., 2010; Xu et al., 2013) have been observed following BPA administration suggesting that there are several possible mechanisms for this effect.

Sex differences in spine density on basal dendrites in CA1 pyramidal cells were observed and females had greater spine density than males, regardless of age. Sex differences were also observed in spine density on basal dendrites of pyramidal cells in the mPFC but, unlike in CA1, the mPFC sex differences interacted with both age and treatment. A sex difference was apparent at 7, but not 11 weeks. Furthermore, the current findings demonstrate that sex interacts with BPA exposure. Specifically, BPA decreases in spine density on basal dendrites in the mPFC were larger in males than in females and this is similar to findings previously reported in the CA1 region of adult mice (Xu et al., 2013). It has previously been shown that BPA leads to decreases in synaptic density, synaptic proteins and glutamate receptors in CA1 of adult male, but not female, rats (Xu et al., 2013) and this was speculated to be due to endogenous estrogen. Consideration must also be given to intrahipppocampal synthesis of estrogen (for review, Hojo et al., 2011). The current data provides novel evidence that the increased male susceptibility to BPA (as previously seen in hippocampal synaptic plasticity measures, Xu et al., 2013) can be extended to include spine density, the period of adolescence, and the mPFC.

We have previously demonstrated that adolescent BPA exposure impairs spatial memory performance in an object placement task in both male and female rats when tested in adolescence (Diaz-Weinstein et al., 2013). Others have shown that perinatal BPA exposure impairs spatial learning and memory on the Morris Water Maze at both postnatal days 21 and 56 (Xu et al., 2010) and it is known that various aspects of these behaviors are mediated by CA1 and mPFC (Broadbent et al., 2004; Jo et al., 2007). Thus, it is appealing to speculate that the observed morphological differences may be contributing to previously reported behavioral effects in response to adolescent BPA exposure. Thus, BPA's effects on spatial memory may be due to dendritic spine density changes. The hippocampus, which plays a vital role in spatial memory and cognition, has many sexually dimorphic features and continues to be responsive to sex hormones through adulthood (reviewed in Hajszan and Leranth, 2010). Since BPA has been shown to have estrogenic and anti-androgenic effects (Jolly, 2009), exposure to BPA during adolescence may effect spatial cognition by altering neural plasticity in the hippocampus.

It is important to note that while stressful experiences can alter dendritic spines, we believe that the dendritic spine density measured in the current study reflect changes due to adolescent BPA exposure and not the 1 h restraint stress exposure for several key reasons. First, all groups experienced the acute stress, and thus alterations in spine density should reflect the differences among groups due to sex, treatment, and age effects. And, the spine density decreases were only observed in the BPA, but not control, groups. Importantly, these findings (e.g., BPA decreases in spine density) are consistent with previously reported data regarding BPAs effect on spine density (Eilim- Stock et al., 2012; Leranth et al., 2008a,b; McLusky et al., 2005b; Xu et al., 2013) ; which further strengthens the argument that the spine density changes were due to the BPA, and not stress, exposure. Second, the vast majority of studies demonstrating stress induced alterations in neuronal morphology have focused on chronic stress exposure (Galea et al., 1997; Magarinos and McEwen, 1995; Sandi et al., 2003; Stewart et al., 2005; Watanabe et al., 1992; for review, Bowman et al., 2003). While changes in spine density following acute stress have been reported (Chen et al., 2008; Sebastian et al., 2013; Shors et al., 2001) these changes did not occur immediately and alterations in CA1 dendritic spine density in male or female rats only occurred after 24 hr (Shor et al., 2001) well outside the timeframe of stress and sacrifice in our study. Third, the primary target of corticosterone influenced neuronal changes is the CA3 region of the hippocampus (Galea et al., 1997; Watanabe et al., 1992), which was not examined in the present study. While stress-induced changes in morphology have been observed in mPFC and CA1 they are not consistent in design with the data presented here. For example, a reorganization of apical arbors in mPFC has been reported following chronic corticosterone administration (Wellman, 2001) or chronic restraint stress (Radley et al., 2004). CA1 dendritic spine density is altered by exposure to intermittent tail shocks (Shors et al., 2001), but this acute stressor places a higher physical demand on the animal than restraint stress (for review, Bowman et al., 2003). In sum, with respect to the 1 h restraint stress challenge, the design of the current study was to examine potential alterations in stress-induced corticosterone levels in adolescent BPA exposed subjects compared to controls and we did not expect there to be any spine density changes due to stress. Finally, it should be noted that the decreased spine densities observed in the present study were not the result of increased dendritic length as we compared the dendritic lengths across all groups for both 7 and 11 weeks and found there to be no effect of BPA on dendritic length in either CA1 or the PFC ($P>0.05$). We recognize that the effects of BPA and stress exposure in the current study are hard to differentiate. BPA exposure could alter the stress response which in turn could have a potential effect on spine density. Future studies are necessary to address this possibility.

Our results demonstrate that adolescent BPA exposure did not alter corticosterone levels following a restraint stress challenge. Previous findings have shown that basal corticosterone levels were increased in neonatally BPA exposed females, but not males, during adolescence (Poimenova et al., 2010). Adolescent sex differences in stress-induced corticosterone release appear dependent upon the stressor. Corticosterone release was greater in BPA treated males than females (Panagiotidou et al., 2014) following an acute forced swim stressor; however, both male and female BPA treated subjects showed elevated corticosterone following the mild Y-maze stressor (Poimenova et al., 2010). Two important distinctions must be considered when making comparisons between the previous and current studies. First, is the

type of stressor and second, is the timing of BPA exposure. Poimenova et al. (2010) and Panagiotidou et al. (2014) exposed rats prenatally to BPA, thus it appears that BPA interferes with the organization of the stress response/hypothalamic-pituitary-adrenal axis during development but the current data suggests that BPA does not exert effects on stress dependent release of corticosterone during adolescence. However, further studies are needed to measure basal corticosterone levels and stress induced corticosterone levels over time following BPA treatment.

While corticosterone levels were not altered by BPA exposure, there were increases associated with age and this effect was greatest for females. These findings are consistent with past reports that showed basal corticosterone levels increased with age (for review, Sapolsky, 1992) and that it is markedly observed in female rats (Lo et al., 1999). Importantly, female corticosterone levels fluctuate across the estrous cycle and, thus it is reasonable to suggest that the age related increases in female corticosterone reported here are due to the onset of estrous cyclicity. Additionally, stress-induced corticosterone release is highest in females during proestrous (Viau and Meaney, 1991) suggesting that future studies should take into consideration the relationship between adolescent BPA exposure and estrous cycle in adulthood.

In conclusion, short term, low dose BPA exposure during the critical period of adolescent development decreased spine density in both basal and apical dendrites in pyramidal neurons of the mPFC and CA1, but did not alter corticosterone release following a 1 h restraint stress challenge. The impact of BPA exposure on spine density was maintained across time and endured into adulthood in both the mPFC and CA1. Furthermore, to the best of our knowledge, we are the first to report that BPA exposure has a significantly greater effect in males than females with regard to spine density decreases in basal dendrites in the mPFC. Importantly, these robust neuronal alterations were observed in animals exposed to BPA levels below the current recommended safe daily limit (United States Environmental Protection Agency, 1993). Humans are routinely exposed to low levels of BPA through a variety of sources and an important consideration when interpreting the current data is the subcutaneous method of BPA administration used in the current study in comparison to an oral route of exposure. No differences were found in circulating BPA levels in neonatal mice following oral or subcutaneous exposure (Taylor et al., 2008); however, future studies should investigate BPA's effects following various routes of administration. Recent studies of Americans showed that 95% had detectable levels of BPA in their urine (Calafat et al., 2005, 2008); thus it seems imperative to better understand BPAs effects on behaviors as well as the possible neuronal underpinnings of these effects.

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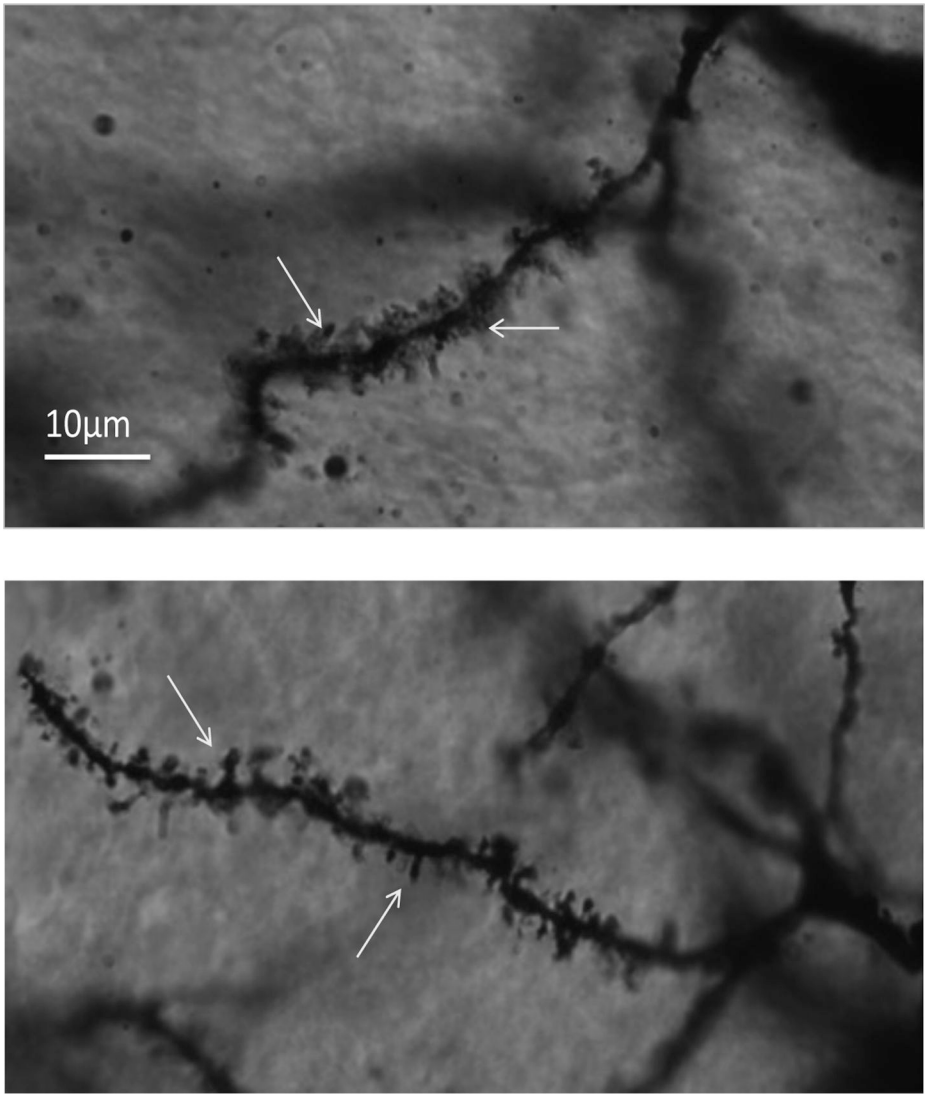

Figure 1.
Photomicrograph illustrating golgi impregnated secondary basal dendrites from pyramidal cells in the mPFC (100 x oil). Top: Control, Bottom: BPA. Arrows denote spines.

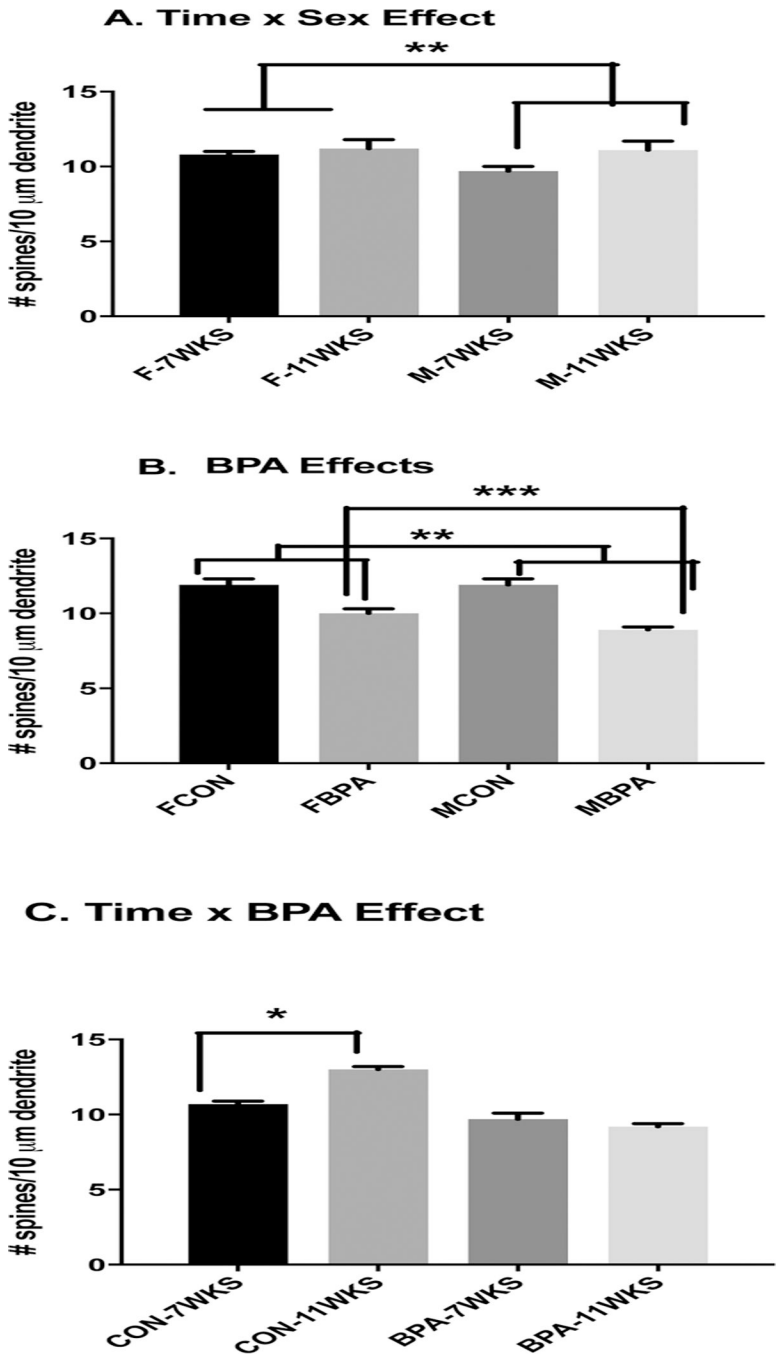

Figure 2.

Pyramidal cells, basal dendritic spine density, mPFC. Entries are the average # spines/10 μm ± SEM. All significant effects are $P<0.05$. Panel A shows the main effects of treatment (CON vs BPA), sex (male vs female) and age (7 weeks vs. 11 weeks). Significant differences between groups are denoted by *. Panel B shows the significant treatment X sex interaction where BPA decreases in spine density were greater in males than females. Panel C shows the significant treatment X age interaction which shows spine density increased across time for control subjects, but not BPA treated. Panel D shows the sex X age

interaction. Across time, males, but not females, showed significant increases in spine density from 7 to 11 weeks.

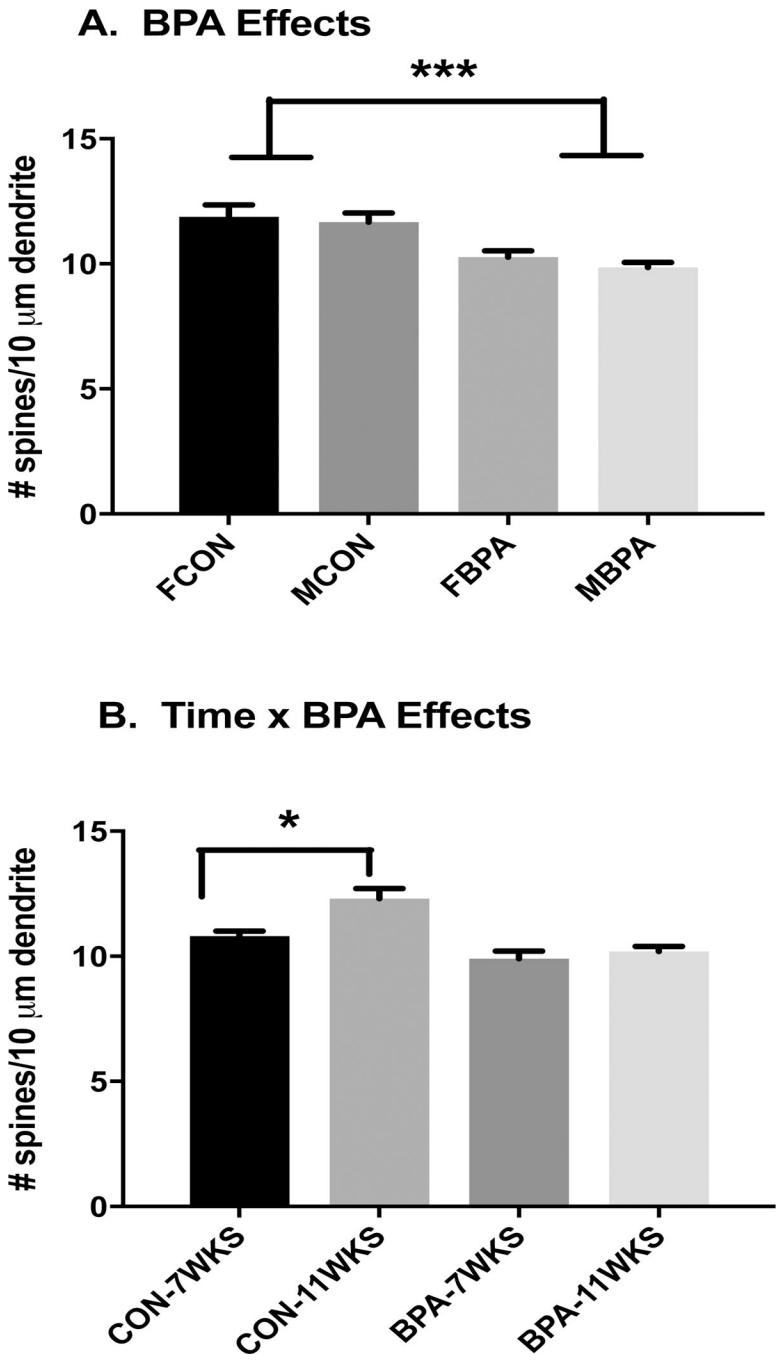

Figure 3.

Pyramidal cells, apical dendritic spine density, mPFC. Entries are the average # spines/10 μm ± SEM. All significant effects are $P<0.05$. Panel A shows the main effects of treatment (CON vs BPA), sex (male vs female) and age (7 weeks vs. 11 weeks). Significant differences between groups are denoted by *. Panel B shows the significant treatment X age interaction. Control subjects increased spine density across time; however, BPA subjects had decreased spine density that persisted from 7 to 11 weeks.

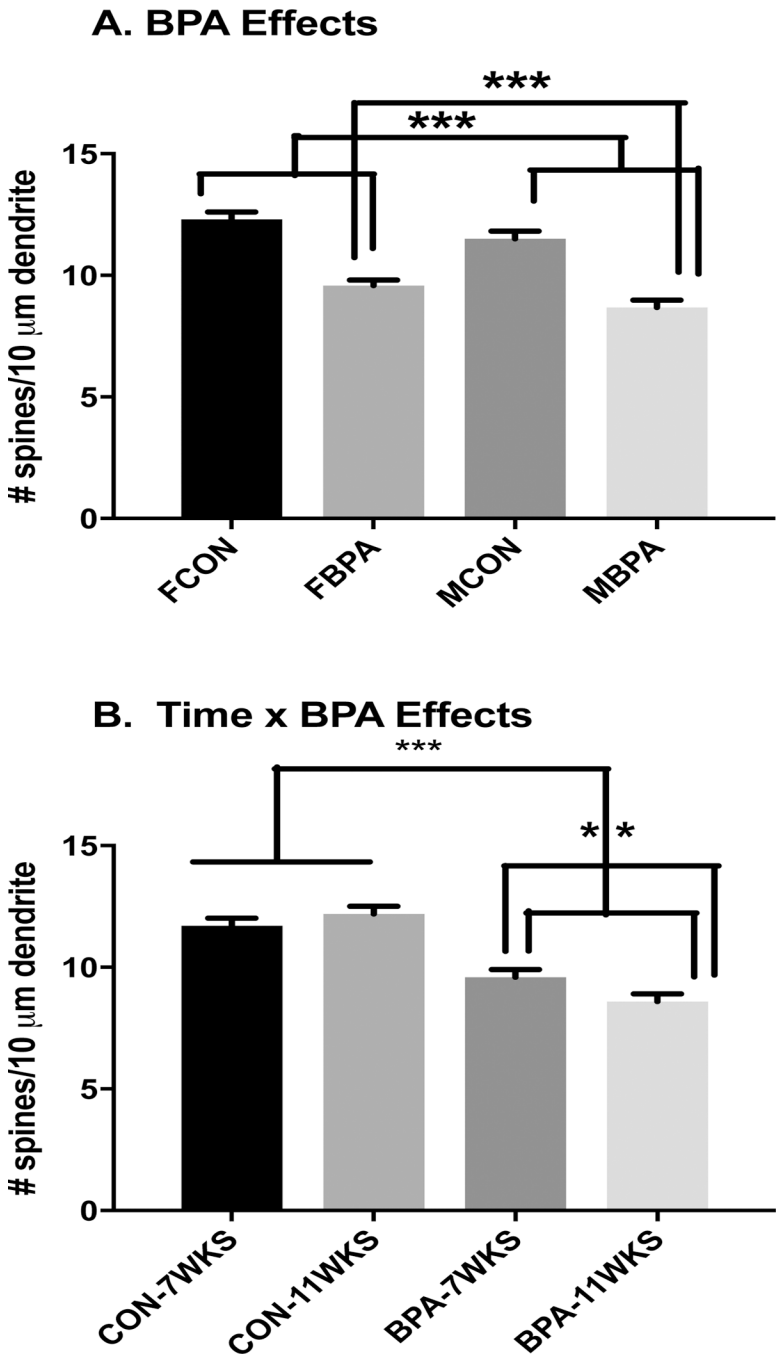

Figure 4.

Pyramidal cells, basal dendritic spine density, CA1. Entries are the average # spines/10 μm ± SEM. All significant effects are P<0.05. Panel A shows the main effects of treatment (CON vs BPA), sex (male vs female) and age (7 weeks vs. 11 weeks). Significant differences between groups are denoted by *. Panel B shows the significant treatment X age interaction. Control subjects had no change in spine density from 7 to 11 weeks; however, BPA treated subjects had a significant decrease in spine density across time.

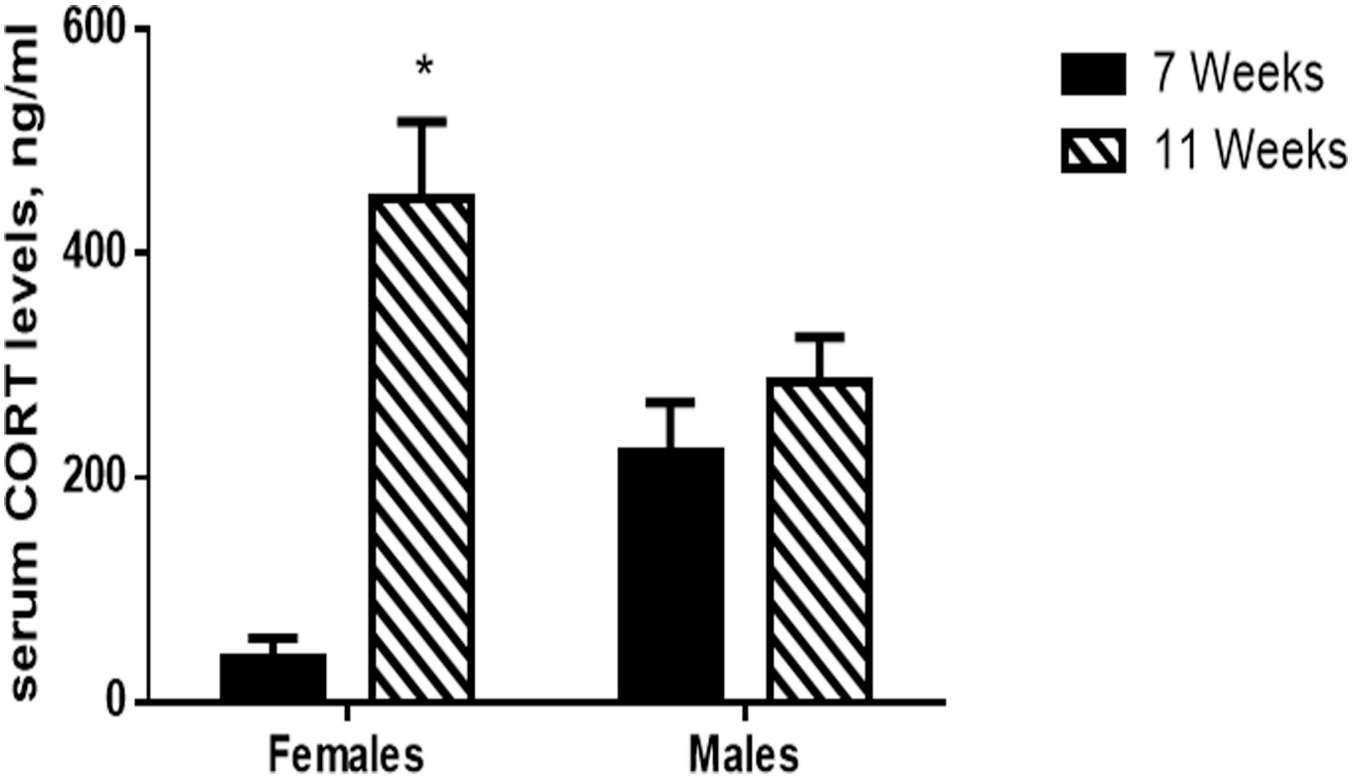

Figure 5.

Corticosterone levels following a 1 h restraint stress challenge. Entries are the serum corticosterone levels, ng/ml, ± SEM. A significant sex X age interaction revealed that females had substantial increases in corticosterone levels from 7 to 11 wks (denoted by *); however, males did not have any significant change in values across time.

Table 1.

Methodological timeline