

Published in final edited form as:

*Neuroscience*. 2014 October 24; 279: 122–131. doi:10.1016/j.neuroscience.2014.08.038.

## Early exposure to bisphenol A alters neuron and glia number in the rat prefrontal cortex of adult males, but not females

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### Abstract

Previous work has shown that exposure to bisphenol A (BPA) during early development can alter sexual differentiation of the brain in rodents, although few studies have examined effects on areas of the brain associated with cognition. The current study examined if developmental BPA exposure alters the total number of neurons and glia in the medial prefrontal cortex (mPFC) in adulthood. Pregnant Long-Evans rats were orally exposed to 0, 4, 40, or 400 µg/kg BPA in corn oil throughout pregnancy. From postnatal days 1-9, pups were given daily oral doses of oil or BPA, at doses corresponding to those given during gestation. Brains were examined in adulthood, and the volume of layers 2/3 and layers 5/6 of the mPFC were parcellated. The density of neurons and glia in these layers was quantified stereologically with the optical disector, and density was multiplied by volume for each animal. Males exposed to 400 µg/kg BPA were found to have increased numbers of neurons and glia in layers 5/6. Although there were no significant effects of BPA in layers 2/3, the pattern of increased neuron number in males exposed to 400 µg/kg BPA was similar to that seen in layers 5/6. No effects of BPA were seen in females or in males exposed to the other doses of BPA. This study indicates that males are more susceptible to the long-lasting effects of BPA on anatomy of the mPFC, an area implicated in neurological disorders.

### Keywords

BPA; prefrontal cortex; neuron number; autism; glia number

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1. Bisphenol A (BPA) is an endocrine disruptor found in a large variety of consumer products, including polycarbonate plastics, resins used to line metal food and drink cans, thermal paper, and certain sealants used in dentistry (Chapin et al., 2008; Richter et al.,

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2007). BPA crosses the blood-brain barrier in adult rats (Sun et al., 2002) and can bind to or act through thyroid, estrogen, and androgen receptors (Kuiper et al., 1998; Moriyama et al., 2002; Zoeller et al., 2005; Sohoni and Sumpter, 1998; Lee et al., 2003). Neural development is an especially vulnerable time for exposure to endocrine disruptors. Neural proliferation and pruning (apoptosis) occur throughout development with region-specific timing (Williams and Herrup, 1988; Kole et al., 2013). Apoptosis during development, in particular, is often influenced by hormones (Simerly, 2002; Bernal, 2005), where an endocrine disruptor, such as BPA, could act. Consequently, it is imperative to determine whether endocrine disruptors, such as BPA, alter neuroanatomical development.

Developmental exposure to BPA alters neuron density or number in subcortical areas, often with a different pattern in males and females (reviewed in Golub et al., 2010; Wolstenholme et al., 2011; Masuo and Ishido, 2012). Many of these studies have concentrated on changes occurring in the hypothalamus in regions that have large sex differences, such as the AVPV (anteroventral periventricular nucleus) and SDN (sexually dimorphic nucleus). For example, at a wide range of doses, developmental BPA exposure decreased tyrosine hydroxylase-positive neuron number in the AVPV in females, while males were either not altered or decreased in this measure in an erratic dose-dependent manner (Rubin et al., 2006; McCaffrey et al., 2013). Additionally, the number of oxytocin-immunoreactive neurons in the AVPV was larger in adult female rats after daily subcutaneous injections of BPA from PND 0-3 (Adewale et al., 2011). After oral exposure to BPA during early development, the volume of the SDN was increased in male rats at PND 21 (He et al., 2012). This contrasts with a decrease in neuron number in the SDN, as measured with Calbindin D28, in adult male rats, while females are unaffected after oral BPA exposure to the dams during early development (McCaffrey et al., 2013).

Only a few studies have concentrated on BPA-induced neural changes outside of the hypothalamus. Oral BPA administration during development (gestation and lactation) resulted in more neurons in males and fewer in females in the locus coeruleus, which reversed the sex difference found in controls (Kubo et al., 2001). Additionally, oral BPA exposure during development increased the density of corticotropin-releasing hormone immunoreactive neurons in the bed nucleus of the stria terminalis in males and decreased them in females (Funabashi et al., 2004). Lastly, oral BPA given during gestation and lactation decreased the density of NeuN-positive neurons in the hippocampus at 20 days of age in males and females (Kunz et al., 2011). However, a decrease in density can be due to an increase in the size of the dendritic tree or other surrounding neuropil as readily as a change in the number of neurons (Boyce et al., 2010). Thus, the potential influence of developmental exposure to BPA on the number of neurons in neural areas directly involved in cognition remains to be investigated.

The rat prefrontal cortex (PFC) is a brain area important for many executive functions including working memory, attention, set-shifting, reversal learning and social learning (Chudasama, 2011; Uylings et al., 2003). In humans, dysfunction of this brain area has been associated with numerous neurological disorders including autism, drug addiction, schizophrenia, and disorders of attention, mood, and anxiety (Hoftman and Lewis, 2011; Price and Drevets, 2012; Teffer and Semendeferi, 2012; Van den Oever et al., 2010). There

have not been any published studies of the effects of developmental exposure to BPA on cognitive tasks which rely heavily in the mPFC (e.g. delayed alternation), but studies testing complex mazes (the radial arm maze or Barnes maze) that have some reliance on the mPFC have failed to find significant effects of developmental BPA exposure (Ferguson et al., 2012; Ryan and Vandenberg, 2006; Sadowski et al., 2014). However, there is substantial evidence that anxiety behaviors, which are sensitive to disruptions of the PFC, are altered by developmental exposure to BPA (Patisaul and Bateman, 2008; Cox et al., 2010; Ryan and Vandenberg, 2006; Adriani et al., 2003; Gioiosa et al., 2007; Rubin et al., 2006; Fujimoto et al., 2006; Kubo et al., 2001; 2003). A recent report has also found an association between neuroimmune activation in the PFC and anxiety-related behavioral changes (Luo et al., in press). Therefore, neuroanatomical changes to the PFC may result in alterations in behavior and could increase the susceptibility to a wide range of developmental and neurological disorders.

Sex differences in brain development and the involvement of gonadal hormones in mediating these differences provide a foundation for the hypothesis that early exposure to endocrine disruptors, such as BPA, could perturb cortical neuron number in adulthood. A previous study found that adult male rats have more neurons in the mPFC compared to females (Markham et al., 2007). Endocrine disruptors could potentially alter neuron proliferation, which occurs prenatally in the rat cortex (Angevine and Sidman, 1962; Miller, 1985). However, there is no evidence that gonadal hormones influence proliferation in the cortex during early development, and studies of non-cortical brain regions, such as the hypothalamus and amygdala, have concluded that developmental estrogen does not impact this process (Jacobson and Gorski, 1981; Park et al., 1996; Wang et al., 2003). Nonetheless, a deficiency in maternal thyroid hormone disrupts fetal neurogenesis in the cortex (Mohan et al., 2012), which is a potential mechanism for how endocrine disruptors could alter adult neuron number.

Apoptosis during development could also be influenced by hormones, and disruptions could alter cell number. Apoptosis continues into the postnatal period in rats, especially during the first 10 days after birth (Ferrer et al., 1990; Nunez et al., 2001). Sex differences in the peaks of cell death in the posterior cortex can be altered by gonadal hormones (Nunez et al., 2001; 2002). The anterior cortex (i.e., the future mPFC) has not been studied in the detail that the posterior cortex has, but a study that broadly examined cell death in the rat cortex found that it was ubiquitous throughout the cortex during the first 10 days with some timing differences among cortical areas (Ferrer et al., 1990). Although Ferrer and colleagues did not explicitly examine the mPFC, it is not unreasonable to assume that apoptotic events occur during the early postnatal period. In addition, the expression of the estrogen receptor  $\alpha$  gene has been found before PND 10 in the mPFC (Westberry and Wilson, 2012). Thus, developmental exposure to endocrine disruptors, such as BPA, could alter proliferation or neuronal pruning and ultimately the total number of neurons. Furthermore, it is likely that this could happen in a sex-dependent pattern. The current study investigates whether perinatal BPA exposure differentially alters adult neuron number in the mPFC of male and female rats.

The number of glia could also be altered in response to environmental exposure to BPA, given that sex differences in total glia number in the mPFC are seen in adulthood (Markham

et al., 2007). GFAP (glial fibrillary acidic protein) expression increases *in vitro* after BPA application and in the hippocampus after developmental exposure to BPA (Kunz et al., 2011; Miyatake et al., 2006; Yamaguchi et al., 2006). Potential effects of BPA have not been examined for microglia or oligodendrocyte number. Therefore, potential effects of BPA on glia in the developing mPFC are merited.

In addition to mediating differences in cell number, gonadal hormones are also implicated in sex-specific development of white matter. Adult males have a larger volume of frontal white matter compared to females (Markham et al., 2007). Also, the splenium of the corpus callosum is larger in males than females, and administration of the estrogen receptor blocker, tamoxifen, or testosterone during early development to female rats increases the size of the corpus callosum in females (Bimonte et al., 2000; Fitch et al., 1990). This indicates that the cortical white matter may be affected by early exposure to BPA.

The present study quantifies the number of neurons and glia in the mPFC and the volume of the underlying white matter after prenatal and early postnatal exposure to BPA. We hypothesize that the role of gonadal influences in the developing mPFC will be disrupted by BPA and result in lasting changes in cortical structure.

## 2. Experimental Procedures

### 2.1 Breeding and Housing Procedures

Two cohorts (separated by approximately 1 year) of adult female and male Long Evans rats from Harlan (Indianapolis, IN) were used as breeders. Precautions were taken to minimize exposure to environmental sources of BPA or exogenous estrogens. Both breeders and their offspring were housed in polysulfone cages (except during mating). A total of 84 offspring born from 42 male and female breeding pairs were used. Rats were given access to reverse osmosis water in glass bottles and were fed a diet from Harlan (2020X) that contains low, but stable amounts of phytoestrogens throughout the experiment. All other materials used in this study (pipette tips, boxes that temporarily held cookies for dosing, etc.) were screened to ensure that they were not composed of materials that could contain BPA. All animal procedures were in compliance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

In order to acclimate female breeders to future dosing procedures, they were handled at least 3 times and fed ½ of a cookie (Newman's Own Arrowroot flavor, Westport, CT) that contained 100 µl of tocopherol-stripped corn oil (Sigma, St. Louis, MO). This method of oral administration has been used in other studies examining neural and behavioral effects of gestational exposure to BPA as well as other endocrine disruptors (McCaffrey et al., 2013; Poon et al., 2013; 2011). For breeding, each male was paired with one female in a wire mesh cage that was monitored daily for the presence of at least one sperm plug underneath the cage.

Three doses of BPA within the µg/kg range were used for several reasons. The current “safe” reference dose of BPA adopted by both the EPA and FDA is 50 µg/kg/day. The

amount of BPA exposure in humans has been highly debated, but estimated exposure has been stated as between 34 ng/kg/day – 100 µg/kg/day (Kang et al., 2006; Lakind and Naiman, 2011; Thomson et al., 2003; Chapin et al., 2008). It is important to note that most of these estimates were based on non-pregnant adult humans, but there is evidence that elevated levels of BPA metabolites have been reported in women who are pregnant, compared with their pre-pregnancy levels (Braun et al., 2012). While most human exposure is thought to fall below 400 µg/kg, this dose as an oral bolus in rodents results in estimated blood levels equal those that are reported in humans (reviewed in Vandenberg et al., 2013). The wide range of estimated exposures in humans and evidence that BPA may exert nonmonotonic effects in rodents (see Vandenberg et al., 2009) was the basis for the use of several doses of BPA (0, 4, 40, or 400 µg/kg/day) in the current study.

## 2.2 Dosing Procedure

On the first day that sperm plugs were found, females were placed in polysulfone cages, and dosing with BPA solutions began. Dosing of the pregnant rats occurred once per day starting at gestational day 0 and continued until parturition. Solutions given to pregnant females were made by suspending BPA powder (obtained from the EPA) in tocopherol-stripped corn oil at concentrations of 0, 0.01, 0.1, or 1 mg BPA/ml, which corresponded to daily doses of 0, 4, 40, or 400 µg BPA/kg of body weight/day, respectively. These solutions were mixed daily with a stir bar prior to dosing to ensure homogeneity. Females were weighed and 0.4 µl of the appropriate solution/g of body weight was pipetted on ½ of a cookie that was allowed to dry. The dams were observed until they finished their cookies, which always occurred within 5 min. The experimenter was blind to all treatments.

BPA was not administered on the day of parturition, which was designated as postnatal day (PND) 0. Doses corresponding to those given to the pregnant females were given by direct oral administration to the pups from PND 1-9 (0, 4, 40, and 400 µg BPA/kg of body weight/day). Solutions of 0, 0.002, 0.02, and 0.2 mg BPA/ml of oil were mixed daily, and a final volume of 2 µl/g of body weight was pipetted into the mouths of the pups while the dam was held temporarily in a separate cage. Oral dosing by insertion of the oil into the mouth has been used in other studies administering BPA to different ages of rodents (Adriani et al., 2003; Delcos et al., 2014; Neese et al., 2013; Wang et al., 2014) and has also been specifically used at this age in rodents for other endocrine disruptors, such as genestein (Cimafranca et al., 2010; Rodriguez-Gomez et al., 2014). This direct oral route mimics oral exposure that occurs in humans and is less stressful than gavage. Although many of the previous studies that examine the effects of early BPA exposure have relied on lactational transfer of BPA from dams to pups as a route of exposure, there is recent evidence to suggest this method results in extremely low exposures to the pups (Doerge et al., 2010).

At PND 2, litters were culled to a maximum of 10 pups, while trying to maintain a balanced sex ratio in each litter. A minimum of 7 pups per litter was required at this time for inclusion in the study. In the one litter that contained less than 7, pups were cross-fostered from litters of the same treatment group and identified by their unique color markings. These cross-fostered pups were not used for later anatomical measures. As reported in a previously published paper, there were no differences in sex ratio between the treatment groups

(Sadowski et al., 2014). At PND 23, all offspring were weaned, double-housed with same-sex littermates, and handled once a week for the remainder of the experiment. Only one male and one female were used per litter for the present study.

### 2.3 Histology

The rats used in current study were tested in a radial arm maze task in adulthood, and the results have been reported in a separate paper (Sadowski et al., 2014). This behavioral experiment required food restriction (85-90% of free-feeding weight) that lasted approximately 5 weeks, and ad libitum food was returned to the rats after completion of the behavioral task. Rats were sacrificed around PND 140, which occurred 2-5 weeks after the rats were returned to free-feeding. This was to ensure that any potential changes to glia due to food restriction and behavioral testing would recover. On the day of sacrifice, rats were injected with 100 mg/kg sodium pentobarbital and perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. All brains were stored in the 4% paraformaldehyde solution for nine days, transferred to a 30% sucrose solution for 3 days, and then coronally sectioned on a freezing microtome. Every fourth 60  $\mu\text{m}$  section was mounted on slides and stained with Methylene Blue/Azure II on the following day.

### 2.4 Cortical and White Matter Volume

On a Zeiss microscope equipped with camera lucida, the ventral mPFC (infralimbic and prelimbic regions) was parcellated based on differences in cytoarchitecture as described previously (Krettek and Price, 1977; Markham et al., 2007; Van Eden and Uylings, 1985). The boundaries of the mPFC within a section, as shown in Figure 1, were established through cytoarchitectonic characteristics of these areas and the cortical regions bordering them. Layer 1 was not included in the analysis since it contains few neurons. The dorsal border of the mPFC is marked by an increase in the density of layer 3 cells, a broadening of layer 5, and a decrease in the thickness of layer 1 that occurs around the transition of the prelimbic region of the mPFC and dorsal anterior cingulate cortex. The infralimbic region of the mPFC is characterized by a relatively uniform lamination pattern and has a less distinct border between layers 1 and 2 compared to the prelimbic region, and ventral boundary was drawn where these characteristics end. The volumes of the prefrontal gray matter and white matter were parcellated from the most anterior section where the white matter first appeared and continued on every mounted section until the appearance of the genu of the corpus callosum, resulting in analysis of 4-6 sections for each brain. Within each parcellation, boundaries were drawn for layers 2/3 and layers 5/6 separately. Within and between cohorts, boundaries were randomly redrawn to confirm consistency within 5% of the original parcellations. Additionally, subcortical white matter underlying the mPFC was traced. All drawings were scanned into a computer, areas were measured with Image J, and post-shrinkage thickness for each layer was measured in the Stereoinvestigator program (Microbrightfield, Williston, VT) to obtain an average thickness. The volumes were calculated with the Cavalieri method as the product of the areas and the tissue thickness between the saved sections with separate calculations for the upper and lower layers of the gray matter.



## 2.5 Neuron and Glia Number

Total numbers of neurons and glia were determined as described previously (Markham et al., 2007; Koss et al., 2012) using the optical disector with the Stereoinvestigator program (Microbrightfield, Williston, VT). Neuron and glia density were quantified separately for layers 2/3 and layers 5/6 of the mPFC. The computer program randomly chose counting frames, which measured  $35\ \mu\text{m} \times 35\ \mu\text{m}$  (width  $\times$  height), within parcellated boundaries. Guard zones were set at  $1\ \mu\text{m}$  at the top and bottom of each section. The investigator counted a cell only if the bottom of the cell was within the volume of the counting frame. Shown in Figure 2, neurons and glia were distinguished based on differences in morphology, size, and color (Markham et al., 2007). In the cortex, neurons are larger and are stained dark blue with a clearly defined nucleus and nucleolus. In contrast, glia are smaller, stain a turquoise blue, and have an amorphous shape. This method of distinguishing between neurons and glia in Nissl-stained sections has been used in studies from several laboratories (Benes et al., 2001; Davanlou and Smith, 2004; Day-Wilson et al., 2006; Rubinow and Juraska, 2009; Markham et al., 2007). At least 400 neurons and 140 glia were counted from layers 2/3 and from layers 5/6 for each animal. These numbers were then divided by the total volume of the counting frames to determine neuron and glia density. The densities were multiplied by the volume of the structure for each animal to determine total number of neurons and glia for individual layers.

## 2.6 Statistical Analysis

Analyses of neuroanatomical measures were performed using ANOVAs (SAS; version 9.4; SAS Institute Inc., Cary, NC) with treatment as a factor and cohort as a covariate because of significant differences between the two cohorts in most measures ( $p < .05$ ). Due to the expectation that males and females may exhibit different responses to BPA, they were analyzed separately. When significant treatment effects were found, a Dunnett's test with treatment as an independent factor and cohort as a covariate was used for posthoc tests to compare individual BPA treatment groups to the control group. This allowed for variance due to cohort to be accounted for in the post hoc comparisons. ANOVAs with sex as the independent factor and cohort as a covariate were also performed between male and female controls to examine potential sex differences. Statistical significance was set at  $p < .05$ . Sample sizes for each sex were controls = 8, 4  $\mu\text{g}/\text{kg}$  = 10, 40  $\mu\text{g}/\text{kg}$  = 13, 400  $\mu\text{g}/\text{kg}$  = 11.

## 3. Results

### 3.1 Neuron Number

There were no significant treatment effects in neuron number in layers 2/3 or layers 5/6 in females (Figure 3A and 3B, respectively; Table 1). In contrast, there was a significant treatment effect in layers 5/6 in males ( $F_{(3,37)} = 3.0$ ;  $p = .04$ ) (Figure 3B). Posthoc tests revealed more neurons in males that received 400  $\mu\text{g}/\text{kg}$  BPA compared to controls ( $D_{(3,37)} = 2.4$ ;  $p < .05$ ). This difference was approximately 15%. The analysis did not reveal a significant effect of treatment in layers 2/3 in males (Figure 3A), but the pattern of increased neuron number in the 400  $\mu\text{g}/\text{kg}$  BPA group was similar to that seen in layers 5/6. Significant cohort effects on this measure were seen in males in layers 2/3 ( $F_{(1,37)} = 13.1$ ;  $p = .001$ ) and 5/6 ( $F_{(1,37)} = 10.7$ ;  $p = .002$ ) and in females in layers 2/3 ( $F_{(1,37)} = 6.2$ ;  $p = .02$ )

and 5/6 ( $F_{(1,37)} = 13.7$ ;  $p = .001$ ). No significant sex differences were detected when control males and females were compared.

### 3.2 Glia Number

There were no significant treatment effects in glia number in layers 2/3 or layers 5/6 in females (Figure 4; Table 1). In contrast, there was significant treatment effect in layers 5/6 in males (Figure 4B) ( $F_{(3,37)} = 2.9$ ;  $p = .046$ ). Post hoc tests revealed significantly more glia in males that received 400  $\mu\text{g}/\text{kg}$  BPA compared to controls ( $D_{(3,37)} = 2.4$ ;  $p < .05$ ). The percent increase was approximately 19%. Analysis did not reveal a significant effect of treatment in layers 2/3 in males (Figure 4A), but the pattern of increased glia number in the 400  $\mu\text{g}/\text{kg}$  BPA group was similar to that seen in layers 5/6. Significant cohort effects on this measure were seen in males in layers 2/3 ( $F_{(1,37)} = 23.8$ ;  $p < .0001$ ) and 5/6 ( $F_{(1,37)} = 35.2$ ;  $p < .0001$ ) and in females in layers 2/3 ( $F_{(1,37)} = 23.1$ ;  $p < .0001$ ) and 5/6 ( $F_{(1,37)} = 19.2$ ;  $p < .0001$ ). No significant sex differences were detected when control males and females were compared.

### 3.3 White Matter Volume

There were no significant treatment effects in the white matter of females (Figure 5; Table 1). There was a significant effect of treatment in the males ( $F_{(3,37)} = 3.1$ ;  $p = .04$ ), but no differences were found between controls and any of the individual treated groups. Significant cohort effects on this measure were seen in males ( $F_{(1,37)} = 9.8$ ;  $p = .003$ ) and in females ( $F_{(1,37)} = 5.3$ ;  $p = .03$ ). Comparisons between control males and females revealed a sex difference ( $F_{(1,13)} = 7.1$ ;  $p = .02$ ) in white matter volume, as reported previously by our laboratory (Markham et al., 2007).

## 4. Discussion

This study provides the first evidence that early exposure to bisphenol A results in a higher number of neurons and glia in the PFC of male rats. This effect was only seen at the highest dose used, 400  $\mu\text{g}/\text{kg}$  BPA. This dose is relevant to prenatal exposure that occurs in humans because it can produce serum blood levels in female adult rodents and female rhesus monkeys that are similar to those observed in female humans (Taylor et al., 2011).

The effect on neuron number in males at the 400  $\mu\text{g}/\text{kg}$  BPA dose was only significant in the lower layers (5/6) of the cortex, although the direction of change in upper layers (2/3) was similar. The number of neurons is especially important because it is formed early in life and is less plastic than other morphological components. Unlike many other alterations, such as those in dendritic spines, glia number or neurotransmitter levels, there are no known pharmacological or environmental interventions (e.g. environmental enrichment) that could be given later in life to normalize neuron number in the cortex. Proliferation of cortical neurons occurs during specific periods of development in contrast to glia, for which proliferation occurs throughout life (Lee et al., 2000).

Mechanisms responsible for the higher number of neurons and glia in males in layers 5/6 at the highest BPA dose are unknown. The timing of the peak of postnatal apoptosis has been shown to differ between male and female rat pups in the developing posterior (visual)



cortex. Males exhibit a higher peak in apoptosis in the early postnatal period (PND 6/7), which corresponds to the time of postnatal BPA exposure in the present study (PND 1-9), while females have higher peaks in apoptosis after PND 10 (Nuñez et al., 2001). The height of these peaks of apoptosis can be altered by early exposure to gonadal hormones. Exposure to 5 $\alpha$ -dihydrotestosterone increased the amount of apoptosis in females to control male values at PND 6 in the posterior cortex (Nuñez et al., 2000). Interestingly, estrogen exposure in females had no effects on this measure. This study establishes that the rate of apoptosis can be altered by gonadal steroids, and it offers the possibility that gonadal hormone receptors are mediating the increase in neuron and glia number in the present study. The hormones involved and the timing in the prefrontal cortex is probably different than in the posterior cortex because differences in the density of androgen receptors have been documented between the posterior cortex and the developing anterior (cingulate/motor) cortex during early development (Nunez et al., 2003). In addition, a recent study reported that early BPA exposure altered estrogen receptor gene expression in the PFC of male and female mice in a dose and sex-specific manner (Kundakovic et al., 2013). Future studies are needed to determine the role of gonadal hormones and their receptors in mediating cell death in the developing mPFC of males and females to elucidate the role of endocrine disruptors.

The animals examined in the current experiment were also behaviorally tested in a radial arm maze as adults, and hormone levels were assessed in their littermates at weaning age (Sadowski et al., 2014). There were no significant changes in thyroxine or thyroid stimulating hormone at weaning in males, suggesting that long-term changes in thyroid hormones are not responsible for the neural changes in males reported here. Similarly, there were no significant changes in reference or working memory observed in any of the groups exposed to BPA. This suggests that the higher number of neurons and glia in the mPFC of 400  $\mu$ g/kg BPA males did not impact radial arm maze behavior. Other studies examining cognitive performance in land-based tasks in rodents after developmental exposure to BPA also found no effects (Ryan and Vandenberg, 2006; Ferguson et al., 2012). However, there is evidence that early exposure to BPA can alter social and anxiety behavior (Adriani et al., 2003; Cox et al., 2010; Dessi-Fulgheri et al., 2002; Gioiosa et al., 2007; Kubo et al., 2001; 2003; Kundakovic et al., 2013; Patisaul and Bateman, 2008; Porrini et al., 2005; Ryan and Vandenberg, 2006; Wolstenholme et al., 2012). The PFC is implicated in modulating social and anxiety behaviors in humans and in animal models (Anderson et al., 1999; Ball et al., 2013; Fossati, 2012; Nelson and Guyer, 2011). Therefore, alterations in the number of neurons and glia in the mPFC in the current study may affect performance on tasks that include emotional and social processing while not affecting other cognitive tasks.

The increases in glia number in the PFC of males exposed to 400  $\mu$ g/kg BPA could be due to changes in astrocytes, oligodendrocytes, or microglia because the Nissl stain employed in the current study does not distinguish between different types of glia. There is limited evidence that GFAP density is acutely increased after exposure to BPA (Yamaguchi et al., 2006; Miyatake et al., 2006; Kunz et al., 2011), but long-term effects are unknown. No studies to date have investigated whether BPA directly impacts oligodendrocytes, but an *in vitro* study did report concentration-dependent alterations in neural progenitor cells and in NG2-positive precursor cells (Okada et al., 2008). However, it is difficult to ascertain how

the concentrations of BPA used in culture studies correspond to doses of BPA used in *in vivo* studies or to exposure that occurs in humans. Therefore, more studies with *in vivo* administration of BPA are needed to determine what types of glia are contributing to the increases seen in this current study.

There is a growing literature that suggests that early exposure to environmental contaminants, specifically those with endocrine-disrupting properties, may be associated with an increased risk of neurological disorders, such as autism. Examples include studies investigating early exposure to phthalates, polybrominated diphenyl ethers, and polychlorinated biphenyls (Larsson et al., 2009; Miodovnik et al., 2011; Mitchell et al., 2012; Testa et al., 2012; Woods et al., 2012). Although the magnitude seen in the present study is relatively small, there is evidence that large increases in neurons and glia are found in human males diagnosed with autism (Courchesne et al. 2011; Edmonson et al., 2014). This suggests that increases in cell number are not necessarily advantageous. It is speculative to suggest that the effects found here could be an indication of a predisposition toward autism, but it does add to the recent examination of the roles that endocrine disruptors may play in the development of neurological disorders (de Cock et al., 2012).

There were main effects of cohort for both the number of glia and neurons. Although we tried to keep all variables the same (sex, strain, testing facility, season, etc. housing conditions), a few of these are not under our control (animal colony conditions and animal caretakers) (Chesler et al, 2002; Sorge et al., 2014). Similar effects of cohort have been reported in other neurotoxicology studies (e.g., Neese et al., 2013) and in some of the variables reported from these animals in Sadowski et al. (2014), most notably in timing of puberty. The cohort effect was consistent across all groups which resulted in the large statistical significance. However, it did not interact with the BPA treatment. The fact that there was a treatment effect in spite of the variability introduced by cohort gives us confidence in our results.

No significant sex differences were seen in either neuron or glia number in the mPFC when male and female controls were compared, despite our previously reported sex differences or indications for sex differences when assessing these measures in adulthood (Markham et al., 2007; Koss et al., 2012). This discrepancy may be due to handling of both the dam during gestation and pups during early postnatal development that was necessary for the administration of the BPA dose. There is evidence for different neural effects between the sexes after early postnatal handling, such as in shock-induced release of adrenocorticotrophic hormone (Erskine et al., 1975), area of the corpus callosum (Berrebi et al., 1988), and turnover of serotonin in PFC (Duchesne et al., 2009). Also, it is unknown how administration of corn oil vehicle during postnatal development impacts sex differences, or neural development in general, given that it is not a natural substance for young lactating rat pups.

In conclusion, exposure to 400  $\mu\text{g}/\text{kg}$  BPA during development resulted in more neurons and glia in the mPFC of male, but not female, rats. The reason for this particular susceptibility in males is unknown, but the binding of BPA to gonadal steroid receptors and differential timing in naturally-occurring cell death between the sexes are possibilities. Future studies

are needed to assess the mechanism for these changes and the specific type of cells that are involved.

## Acknowledgments

We thank Nioka Lowry and Wendy Koss for their assistance. We would also like to thank the Microscopy Suite at the Beckman Institute for support and use of their facilities and Ghazal Naseri Kouzehgarani for help with the statistics. This work was supported by NIEHS P20 ES 018163, EPA RD 83459301 Project 4, NIEHS T32 ES007326.

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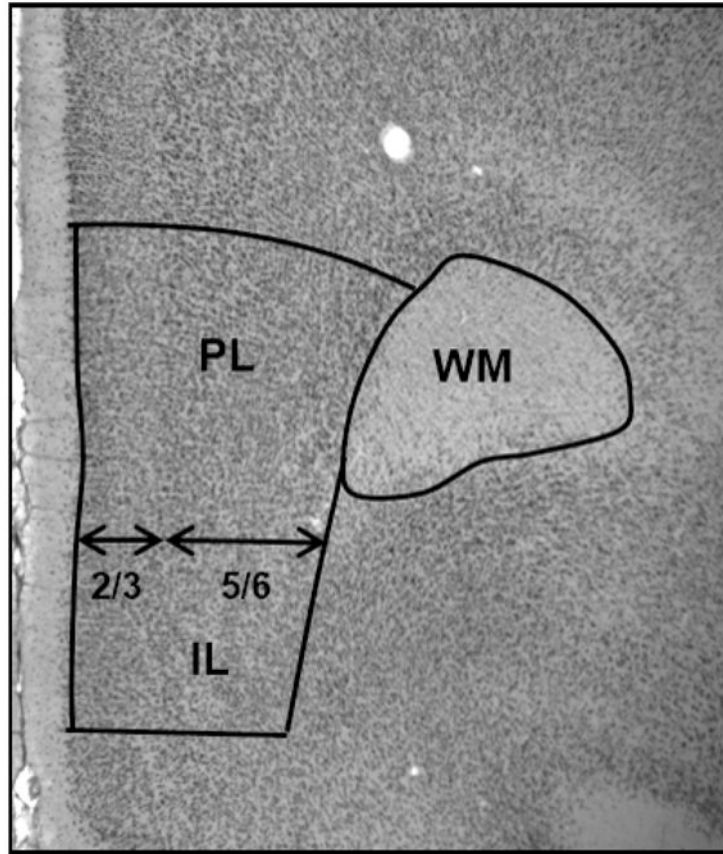
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## Abbreviations

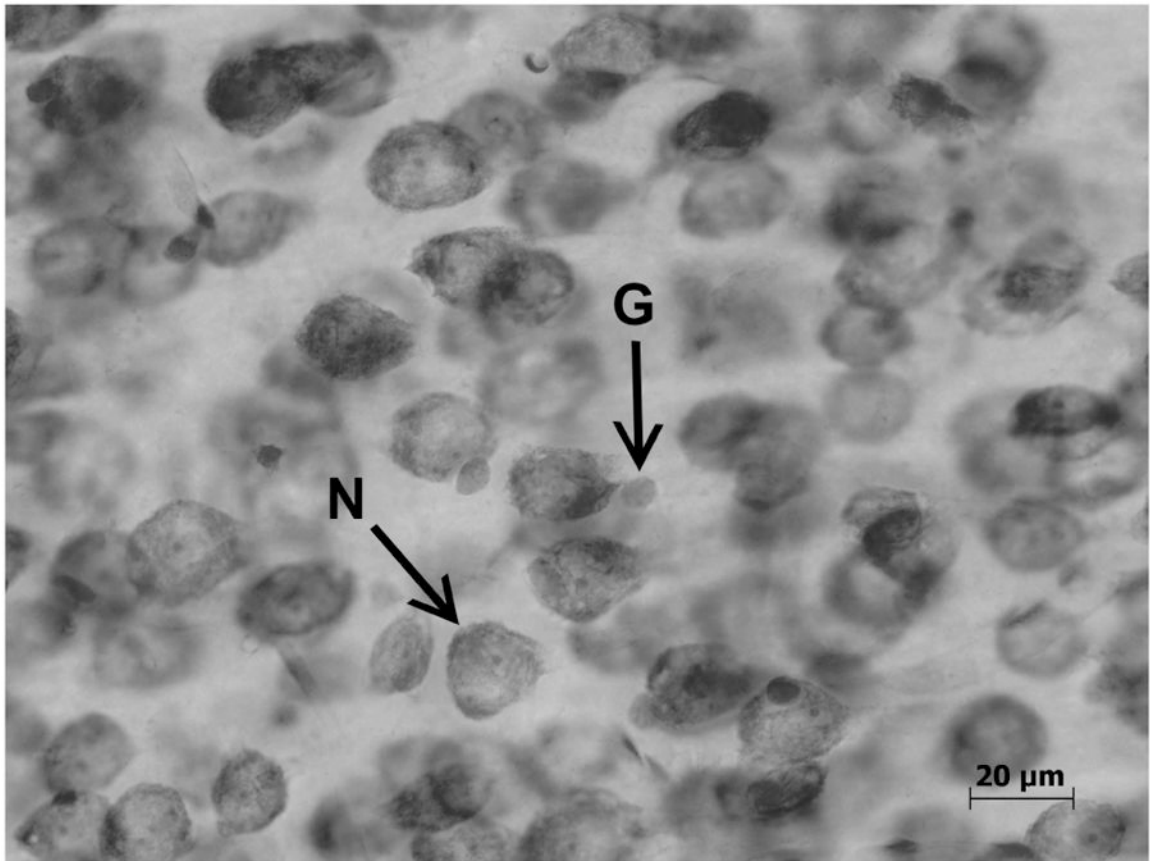
<b>BPA</b>	bisphenol A
<b>mPFC</b>	medial prefrontal cortex
<b>PFC</b>	prefrontal cortex
<b>AVPV</b>	anteroventral periventricular nucleus
<b>PND</b>	postnatal day
<b>GFAP</b>	glial fibrillary acidic protein
<b>SDN</b>	sexually dimorphic nucleus

### Highlights

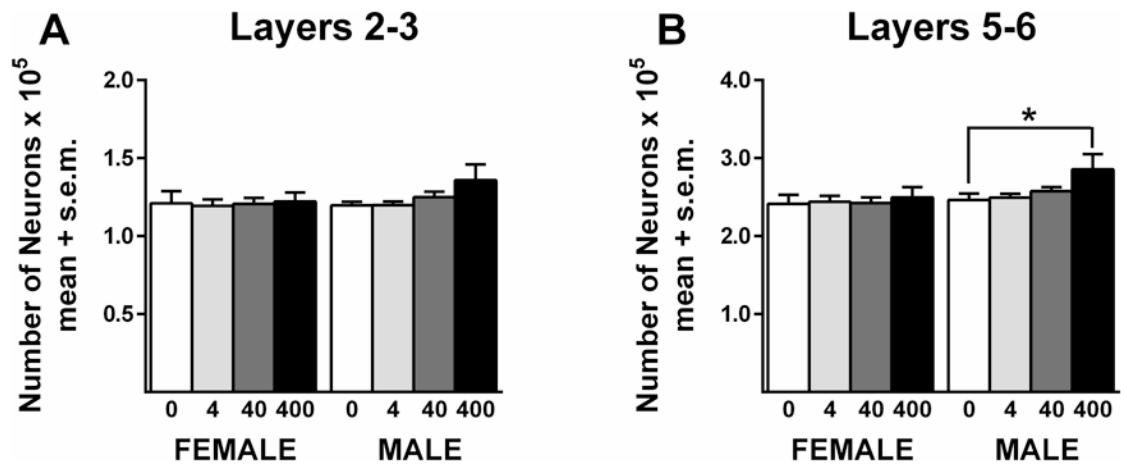
- Pregnant females received 0, 4, 40, or 400  $\mu\text{g}/\text{kg}/\text{day}$  BPA throughout gestation.
- Male and female offspring were orally given the same dose from postnatal days 1-9.
- In adulthood, neuron and glia number in the medial prefrontal cortex were quantified using stereological techniques
- Males exposed to 400  $\mu\text{g}/\text{kg}/\text{day}$  BPA during development had more neurons and glia in layers 5/6 compared to male controls.
- Females were unaffected.



**Figure 1.** A photograph illustrating the boundaries of parcellations for the gray matter of the mPFC and white matter. prelimbic (PL), infralimbic (IL), white matter (WM)



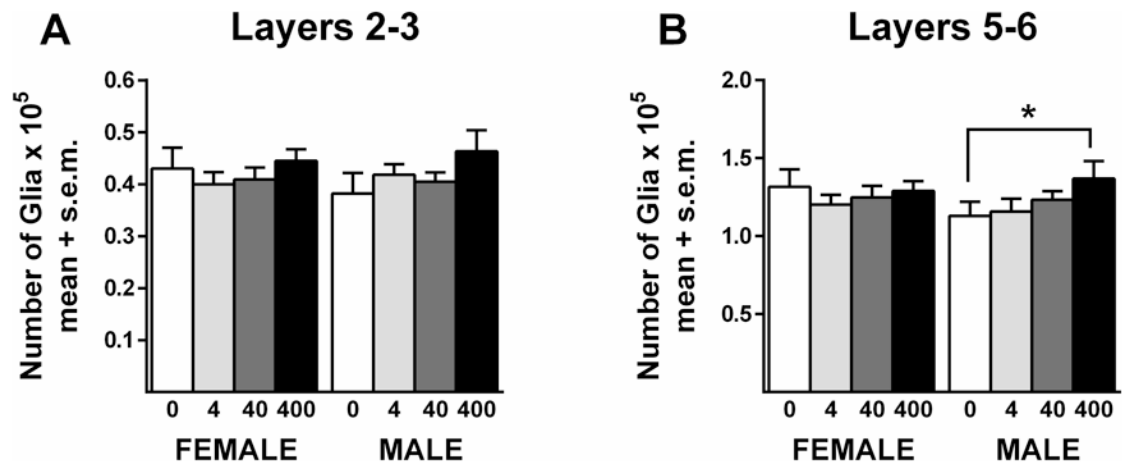
**Figure 2.** A photograph showing a neuron (N) and glia (G) at high magnification. (Koss et al., 2012)



**Figure 3.**

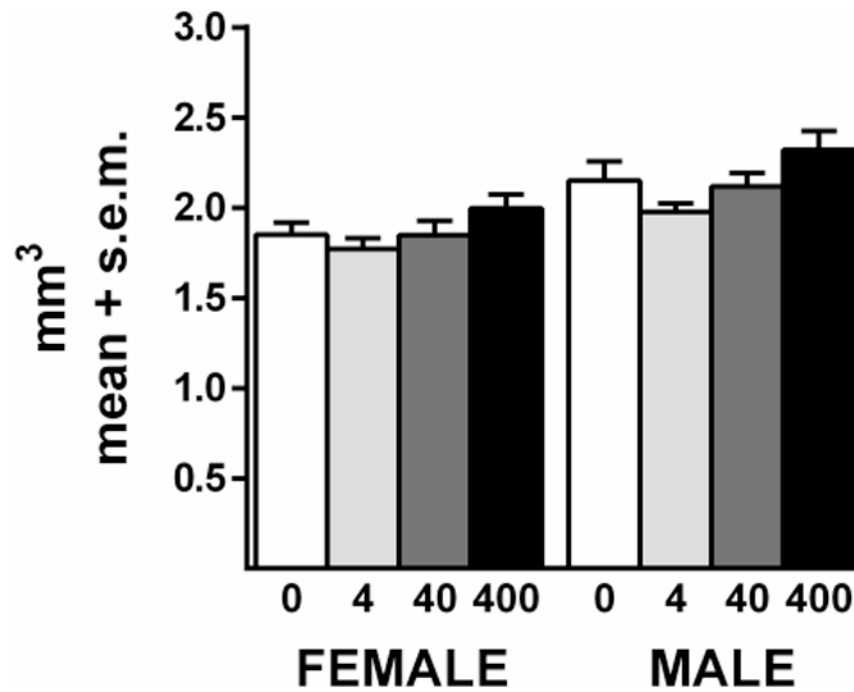
Neuron number in the mPFC. BPA did not alter neuron number in females. There was a significant difference between the controls and the 400 µg/kg BPA in males. \* $p < .05$ . Sample sizes for each sex were controls = 8, 4 µg/kg = 10, 40 µg/kg = 13, 400 µg/kg = 11.





**Figure 4.**

Glia number in the mPFC. BPA did not alter glia number in females. There was a significant difference between the controls and the 400 BPA group in males. \* $p < .05$ . Sample sizes for each sex were controls = 8, 4 µg/kg = 10, 40 µg/kg = 13, 400 µg/kg = 11.



**Figure 5.** White matter volume. BPA did not alter the volume of the white matter in females. In males, there was a significant overall treatment effect ( $p < .05$ ) but no significant differences between controls and individual treatment groups. Sample sizes for each sex were controls = 8, 4  $\mu\text{g}/\text{kg}$  = 10, 40  $\mu\text{g}/\text{kg}$  = 13, 400  $\mu\text{g}/\text{kg}$  = 11.

Table 1

## Summary of Results

Dose ( $\mu\text{g}/\text{kg}$ )	Sex	Neuron Number Layers 2/3 ( $\times 10^5$ )	Neuron Number Layers 5/6 ( $\times 10^5$ )	Glia Number Layers 2/3 ( $\times 10^5$ )	Glia Number Layers 5/6 ( $\times 10^5$ )	White Matter Volume ( $\times 10^6$ )
0	Female	1.21 $\pm$ 0.08	2.41 $\pm$ 0.12	0.43 $\pm$ 0.04	1.32 $\pm$ 0.11	1.85 $\pm$ 0.07
4	Female	1.19 $\pm$ 0.04	2.44 $\pm$ 0.07	0.40 $\pm$ 0.02	1.20 $\pm$ 0.06	1.77 $\pm$ 0.06
40	Female	1.21 $\pm$ 0.04	2.43 $\pm$ 0.07	0.41 $\pm$ 0.02	1.25 $\pm$ 0.07	1.85 $\pm$ 0.08
400	Female	1.22 $\pm$ 0.06	2.50 $\pm$ 0.13	0.44 $\pm$ 0.02	1.29 $\pm$ 0.06	2.00 $\pm$ 0.08
0	Male	1.20 $\pm$ 0.02	2.46 $\pm$ 0.08	0.38 $\pm$ 0.04	1.13 $\pm$ 0.09	2.15 $\pm$ 0.11
4	Male	1.20 $\pm$ 0.02	2.49 $\pm$ 0.05	0.42 $\pm$ 0.02	1.16 $\pm$ 0.08	1.98 $\pm$ 0.05
40	Male	1.25 $\pm$ 0.03	2.58 $\pm$ 0.05	0.40 $\pm$ 0.02	1.23 $\pm$ 0.05	2.12 $\pm$ 0.07
400	Male	1.36 $\pm$ 0.10	2.86 $\pm$ 0.19*	0.46 $\pm$ 0.04	1.37 $\pm$ 0.11*	2.32 $\pm$ 0.07

\* Significant difference from 0 dose male.