EFFECTS OF BISPHENOL A EXPOSURE DURING EARLY DEVELOPMENT ON BEHAVIOR AND ANATOMY OF THE PREFRONTAL CORTEX IN ADULT MALE AND FEMALE RATS.

BY

RENEE NICOLE SADOWSKI

DISSERTATION

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Doctoral Committee:

Professor Janice Juraska, Chair
Professor Susan Schantz
Professor Jodi Flaws
Associate Professor Joshua Gulley
Assistant Professor Megan Mahoney

Abstract

Previous work has shown that exposure to bisphenol A (BPA) during early development can affect behavior and sexual differentiation of the brain in rodents, although few studies have examined effects on cognitive behavior and associated areas of the brain. The current study examined if exposure to BPA during early development alters general measures of growth and development, cognitive behavior, and anatomy of medial prefrontal cortex (mPFC) in adulthood. Long-Evans hooded rats were orally exposed to corn oil (vehicle), 4µg/kg, 40µg/kg, or 400µg/kg throughout pregnancy and during the early postnatal period. Measures of general growth and development, such as body weight, puberty onset, and levels of thyroxine (T4) were assessed at weaning age. One male and one female from each litter were trained on the 17-arm radial arm maze in adulthood. Several weeks following completion of training, brains were removed from these rats to stereologically assess neuron and glia number in the medial prefrontal cortex.

Results indicated that levels of T4 at weaning age were significantly altered by exposure to BPA. Additionally, there were also minor indications for learning impairments in the radial arm maze task in males, although these were only seen in late in training and most comparisons revealed only non-significant trends. For neuroanatomical measures, males exposed to 400ug/kg/day BPA displayed a significantly larger number of neurons and glia in layers 5-6 of the mPFC. In conclusion, exposure to BPA leads to alterations in T4 levels at weaning age and significant changes in anatomy of the prefrontal cortex in males only. The changes in

neuroanatomy of the prefrontal cortex may be indicative of a particular susceptibility of males to exposure of BPA.

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Chapter 1:

Background and Significance

Sources of and Exposure to Bisphenol A in Humans

Bisphenol A (BPA), an endocrine disruptor, is a chemical used in the production of many products including polycarbonate plastics (Biles et al., 1997; Krishnan et al., 1993), resins used to line cans (Brotons et al., 1995), thermal or recycled paper (Biedermann et al., 2010; Ozaki et al., 2004), and certain sealants used in dentistry (Olea et al., 1996). It is estimated that global production of BPA exceeds 6 billion pounds of BPA annually and it is expected that this amount will only increase in the coming years (Burridge, 2003). According to the recent National Health and Nutrition Examination Survey, approximately 93% of the human population shows detectable amounts of BPA in urine (Calafat et al., 2008).

Amid growing concerns of the potential of BPA to disrupt various aspects of anatomy, physiology, and behavior, Canada was the first country to declare BPA as a toxic chemical in 2008. In this same year, U.S. retailers of water and baby bottles began to remove products that contain BPA from their shelves. However, the European Food Safety Authority and the U.S. Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) still consider exposure to BPA at levels estimated to occur in humans as safe. The current “safe” reference dose of BPA adopted by both the EPA and FDA is 50µg/kg/day. This reference dose is based on carcinogenesis studies conducted in the 1980s in which 50mg/kg/day was the lowest observed adverse effect level for decreases in body weights in adult animals given diets

that contained BPA (NTP, 1982; reviewed in Vogel et al., 2009). Then, 50mg/kg/day was divided by 1000 (used as an uncertainty factor) in order to obtain the 50µg/kg/day "safe" reference dose that is still used as the standard by U.S. federal agencies today.

There are significant problems with both the data used to estimate the "safe" dose and the conclusions that are drawn from these data. First, the original study used to determine 50µg/kg/day as the "safe" dose was concluded in 1977 by a private company which was later investigated for poor quality-control and pathological practices. The committee that was appointed by the U.S. Congress to investigate these problems concluded that these issues could have affected the outcomes of any research, including the BPA carcinogenesis studies that occurred prior to the investigation (Hart, 1979). Second, the current "safe" dose is not based on empirical evidence of low dose studies (i.e. those that occur in the human population), but is derived from studies investigating extremely high doses and using the results of one particular end point (which was body weight). Lastly, this conclusion is based on information obtained only in adult animals and did not address the potential of BPA to produce effects when given to animals undergoing significant developmental changes. Of great concern is the fact that BPA has been shown to exhibit nonmotonic dose response curves in both in vitro and in vivo models in rodents (see Vanderberg et al., 2009). Therefore, low doses, or those seen in human exposure, may be hazardous to human health, contrary to the "safe" dose currently accepted by the U.S. FDA and EPA.

Although human exposure to BPA is thought to mainly occur through leaching from products used for food packaging, such as polycarbonate plastics and epoxy resins, the use of BPA in other human-use products is widespread. BPA has been

detected in a vast array of human biological tissues including, but not limited to, maternal blood, maternal urine, neonatal blood, placenta, cord blood, amniotic fluid, and breast milk (Vandenberg et al., 2007; 2010). Exposures to BPA in the population are likely to vary, depending on variations in lifestyle, such as occupation, personal habits, and age. Additionally, estimates of human exposure are also variable depending on the method for determination. Taking into account various data from sources of BPA (food, water, soil, and air), Kang et al. (2006) estimated intake of BPA to be less than 1µg/kg/day, while the European Food Safety Authority (EFSA, 2006) estimates exposure to be approximately 1.5µg/kg/day in adults and 0.2-13µg/kg/day in infants and children just from food sources alone. A study conducted in the United States in 2005-2006 estimated daily intake of BPA based on levels of measured urinary BPA (parent BPA + metabolites) to average around 34ng/kg/day, although children under 6 years of age exhibit higher levels than adults (Lakind & Naiman, 2011). Another approximation of exposures in humans suggested that New Zealanders may be exposed to as much as 4.8µg/day of BPA from food/dietary sources alone (Thomson et al., 2003). Therefore, it is conclusively established that the general population is exposed to BPA, although the specific range of exposure has been highly debated.

Although estimates in the normal population are well below the current “safe” dose used by the FDA and EPA, most of these estimates do not account for exposure from known sources of BPA other than those associated with food consumption. BPA is also transmitted through a transdermal route (Zalko et al., 2011), and it has been estimated that individuals who daily handle thermal paper receipts, such as cashiers, may be exposed to levels up to 71µg/day, which is significantly greater than those

estimated to occur in normal population (Biedermann et al., 2010; Braun et al., 2011). U.S. powder paint workers may be exposed to as much as 100µg/kg/day (NTP, 2008; Chapin et al., 2008). Even more alarming is a recent study that found that infants in the intensive care unit are exposed to higher than normal and varied levels of BPA most likely through the use of medical devices (Calafat et al., 2009). Certain personal practices, such as heating plastics or exposing plastics to high pH environments, can increase the amount of BPA that leaches from containers into food and water (Brede et al., 2003; Krishnan et al., 1993). Lastly, it is also important to note that exposure to BPA is likely increasing, given that the levels of BPA in human urine doubled overall and tripled in the 95th percentile between 1994 and 2004 surveys (NHANES III, 1994; NHANES, 2004).

It is undeniable that further human studies are needed to clarify not only the exposure to BPA in different populations, but also the potential of this chemical to disrupt anatomy, physiology, and behavior. However, several limitations of human and epidemiologic studies must be addressed. First, humans are highly likely to be exposed to a wide variety of other endocrine disruptors, such as phthalates, which may confound results and make it difficult to decipher the specific effects of BPA. Additionally, human studies allow for correlational information about specific toxicants and changes in the brain and behavior, but make it difficult to test direct effects of chemical exposures. For these reasons, it is important to investigate the potential low-dose effects of BPA in animal models, where outside exposure can be better controlled and specific mechanisms that may influence cognition and behavior, such as changes in brain anatomy, can be assessed.

Metabolism of BPA

One important factor in assessing human exposure to potential toxicants is metabolism and pharmacokinetics, which lends insight into how often and to what extent the human population is exposed. However, due to difficulty of assessing metabolism of BPA in humans, most of this work has employed rodent or non-human primate models. One study has suggested that the route of exposure of BPA is likely to have a large impact on metabolism (Pottenger et al., 2000), given that oral exposure would be susceptible to first pass metabolism while other routes, such as subcutaneous injection or transdermal exposure, would not. However, more recent evidence suggests that the impact of route of exposure is dependent on the age of the animal model. Doerge et al., (2011) demonstrated that PND 3 mice showed similar levels of the unconjugated form when BPA was given subcutaneously or orally through gavage. However, differences were seen between the two different exposures when PND 10 or older mice were examined. The authors pointed out that these age-specific effects are most likely due to an underdeveloped liver in the early postnatal life of mice that does not occur during the late fetal stage in non-human primates (Doerge et al., 2011; Doerge et al., 2010a; 2010b).

These species comparisons have led to discussion about the species specificity of metabolism of BPA. The fact that metabolism of BPA in perinatal rodents is different than that of the fetal monkey after administration of BPA by intravenous injection has led to discussions that the rodent model may not always represent the best model for human exposure to BPA. While it has been suggested that adult non-human primates and rodents metabolize BPA in a similar manner (Doerge et al., 2011; Taylor et al.,

2008), there may be metabolic differences between species during the fetal age. However, as levels of unconjugated BPA in serum were not reported in non-human primate fetuses after oral dosing (Doerge et al., 2010b) even though they were reported during early postnatal mice and rats (Doerge et al., 2010a; 2011), it is difficult to make direct comparisons with respect to oral dosing between these different studies.

Liver metabolism of BPA is accomplished mainly through glucuronidation by UDP-glucuronosyltransferase, and BPA-glucuronide has been identified as the major metabolite in humans and rodents (Snyder et al., 2000; Kurebayashi et al., 2003; 2010; Völkel et al., 2002). BPA-glucuronide has been shown to have little or no estrogenic activity in vitro (Matthews et al., 2001), although it has been suggested that other metabolites of BPA, such as MBP, may bind estrogen receptors more potently than the parent compound (Ishibashi et al., 2005; Yoshihara et al., 2004). Parent BPA is mainly excreted through the feces, while the main metabolite, BPA-glucuronide, is excreted primarily through urine (Synder et al., 2000, Pottenger et al, 2000).

Several human studies have reported a fast metabolism of BPA, with a half life, as detected in urine, of between 4-5.5 hours after oral exposure (Völkel et al., 2002; 2005). Based on this information, it is generally concluded that the entire process is complete within 24 hours, making it likely that estimated exposures in humans are a result of persistent exposure to the chemical. However, more recent data suggest that human BPA-glucuronide levels did not decline rapidly after significant fasting, potentially because significant exposure to BPA is occurring either from non-food sources or is accumulating in other tissues, such as fat (Stahlhut et al., 2009). In rodents, a recent study examined metabolism and time courses after oral administration and found that

peak values in plasma are reached between 15-45 minutes after ingestion of BPA (Domoradzki et al., 2004). A second peak of BPA was noted at about 24 hours and levels were still quantifiable after 96 hours. Although it is possible that BPA persists longer than expected as based on levels of metabolites of BPA in human urine, the demonstrated short half life of this chemical along with the pervasive use of BPA in human-use products suggests that human exposure is likely occurring on a persistent and daily basis.

Hormonal Mechanisms of BPA

BPA belongs a group of chemicals that are often referred to as endocrine disruptors, primarily based their ability to bind to estrogen receptors. This action of BPA has been proposed as the mechanism by which BPA may exert effects on reproduction, brain anatomy, and brain function. Many past studies have therefore used estradiol or an estrogen receptor agonist to serve as a positive control. As assessed in animal models, BPA's affinity for estrogen receptor α (ERα) and estrogen receptor β (ERβ) is 1,000-10,000 fold less than estradiol (Gould et al., 1998; Kuiper et al., 1998), and it binds ERβ with a higher affinity than ERα (Kuiper et al., 1997; Matthews et al., 2001; Routledge et al., 2000). One report suggests that in some cell types, BPA may exert mixed agonist and antagonist activity through ERβ (Kurosawa et al., 2002). Some of the initial lack of concern by certain governmental agencies for BPA exposures at human relevant doses grows from the past evidence that BPA is a very weak agonist at estrogen receptors. However, more recent reports suggest that BPA also binds to non-classical membrane estrogen receptors and G-protein coupled receptors (such as GPR30) and has actions through non-genomic pathways (Alonso-Magdalena et al.,

2005; 2011; Zsarnovszky et al., 2005; Watson et al., 2005; Thomas & Dong, 2006; Ropero et al., 2006). Furthermore, BPA likely alters expression of classical estrogen receptors in the brain. Alterations in levels of ERα and ERβ expression (Ramos et al., 2003; Cao et al., 2012), ESR1 and ESR2 mRNA expression (Khurana et al., 2000; Kawai et al., 2007; Cao et al., 2013), and the number of ER-labeled neurons (Ceccarelli et al., 2007) have all been reported in rodent models, although the magnitude and direction of effects are specific to particular brain areas.

In addition to effects relating to estrogen and estrogen receptors, BPA binds to or alters other hormone systems (see Rubin et al., 2011). The most studied of these is thyroid hormone. BPA antagonizes thyroid hormone receptors, although the affinity is lower than that for estrogen receptors (Moriyama et al., 2002; Zoeller et al., 2005). Gestational and lactational exposure to BPA also increases levels of thyroxine and expression of thyroid responsive genes in the brain during early postnatal development in rodents (Zoeller et al., 2005). Furthermore, BPA given during gestation and early postnatal development resulted in a transient hyperthyroidism in a lower dose of BPA (at postnatal day 7) and hypothyroidism in a higher dose of BPA (at postnatal day 21) in male offspring, while no effects were seen in female offspring (Xu et al., 2007).

Estrogen related receptor gamma (ERRγ) is another receptor to which BPA binds with a relatively high affinity (Matsushima et al., 2008; Okada et al, 2008). Importantly, there is high expression of ERRγ in the placenta, potentially contributing to accumulation of BPA in the placenta and effects of BPA on early development (Takeda et al., 2009; Arase et al., 2011). Lastly, androgen receptors are another potential target of BPA. Several *in vitro* studies suggest that BPA has antiandrogenic activities (Sohoni

& Sumpter, 1998; Lee et al., 2003; Sun et al., 2006; Kruger et al., 2008), and in addition can inhibit aromatase (Bonefeld-Jorgensen et al., 2007), an enzyme that mediates the conversion of testosterone to estrogen.

Increased Susceptibility to BPA During Early Development

Although the majority of the human population is exposed to BPA, particular concern has arisen regarding the potential effects of exposure to BPA during early development. The fetus and neonate are normally protected from the impacts of estrogens by α-fetal protein, which prevents estradiol from passing through the placenta. However, it is thought that BPA binds serum proteins with limited capacity compared to other estrogens (Milligan et al., 1998). Elevated levels of BPA metabolites have been reported in women who are pregnant, compared with their pre-pregnancy levels (Braun et al., 2012). Also, BPA readily passes through the placenta, mostly in its active unconjugated form (Balakrishnan et al., 2010). Therefore, the fetus and neonate may be particularly susceptible to being exposed to parent BPA. Past investigations that assess levels of BPA in maternal compared to fetal tissues also support the idea that the fetus is exposed to BPA. In humans, levels of BPA detected in the amniotic fluid were approximately 5 times higher than those reported in other fluids, such as the serum from pregnant women (Ikezuki et al., 2002). Additionally, median levels of BPA were 3.1ng/ml in maternal plasma, 2.3ng/ml in fetal plasma, and 12.7ng/g in placental tissue (Schönfelder et al., 2002). In rodents, BPA was administered to pregnant females and was detected in rodent fetal tissue at levels greater than those detected in maternal blood, and BPA was still detectable up to 50 hours after exposure, the time at which the measurement was terminated (Takahashi & Oishi, 2000). BPA is found in

higher concentrations in the mouse fetus and amniotic fluid than in maternal blood during maternal exposure of BPA (Zalko et al., 2003). Therefore, BPA is likely passed from the mother to the developing fetus in both humans and rodents.

While much of the previous information on the metabolism of BPA discussed in the preceding section explained general metabolism of BPA, differences in metabolism during pregnancy or within the developing fetus are of particular concern. A recent relevant study found that the fetal liver has no UDP-glucuronosyltransferase activities, leading to the conclusion that the fetal rat is unable to metabolize BPA in the same way as adults (Matsumoto et al., 2002). The same study reported that rat hepatic microsomal UDP-glucuronosyltransferase activities were decreased 2 fold in pregnant rats. Therefore, exposure to parent BPA is likely higher in the fetus than previously thought due to the fact that a significant amount of the studies that assess metabolism of BPA are based solely in non-pregnant adult rats.

Effects of BPA Sexual Behavior and Reproduction

Some research regarding BPA has focused on effects on reproduction and reproductive tissues. There has been evidence that BPA, at certain doses, alters aspects of sexual behavior in male rodents. For example, sexually experienced males exposed to 50µg/kg/day had decreased number of intromissions and copulatory efficiency (# of intromissions/# of mounts) and increased ejaculation latency compared to controls (Jones et al., 2011). Interestingly, neither 5µg/kg/day nor 500µg/kg/day significantly altered these sexual behaviors and in fact, those given 5mg/kg/day actually displayed enhanced sexual behavior, as evident by decreased post-ejaculatory, mount, and intromission intervals in a sexually inexperienced condition. In addition, almost all

aspects of male sexual behavior examined in this study (Jones et al., 2011) showed nonmotonic dose response curves with respect to BPA. In another study examining male sexual behavior and using only one dose of BPA (40µg/kg/day), males were impaired in both the intromission latency and number of intromissions (Farabollini et al., 2002). Studies assessing sexual behavior in females have reported mixed findings, with evidence for impaired (Monje et al., 2009), enhanced (Jones et al., 2011; Farabollini et al., 2002), or unaffected (Ryan et al., 2010; Adewale et al., 2009) receptive or proceptive behaviors.

Various measures of reproductive function in females and males suggest a susceptibility to BPA during early development (for review see Salian et al., 2011; Richter et al., 2007). For instance, fecundity may be impaired in females exposed during gestation and lactation, as demonstrated in a forced breeding experiment in which the cumulative number of pups was decreased in groups receiving either 25ng/kg/day or 25µg/kg/day, and the total number of litters was decreased in the group receiving 25µg/kg/day (Cabaton et al., 2010). Extensive evidence is available for studies in rodents in which changes were detected in the reproductive tract due to BPA exposure. In females, alterations in the anatomy or physiology of the vagina, uterus, and ovary have all been reported (Markey et al., 2005; Schönfelder et al., 2002; 2004; Nikaido et al., 2004; Peretz et al., 2011). Although two studies have failed to provide any evidence for changes in general fertility in males after developmental BPA exposure (Ema et al., 2001; Tyl et al., 2002), it is highly probable that BPA may affect reproductive measures such as spermatogenesis, sperm motility, and sperm

morphology (Salian et al., 2009; Toyama et al., 2004; Toyama & Yuasa, 2004; Aikawa et al., 2004).

Effects of BPA on the Brain and Behavior

Currently, one of the largest concerns for BPA exposure during development is its impacts on the brain and behavior. Long-lasting changes due to early exposure of BPA have been noted in a variety of tasks including those that measure anxiety, sexual, learning and memory, and social behavior. In addition, changes in the development of brain areas associated with reproductive and cognitive abilities have been found with respect to BPA. A number of recent papers have reviewed both behavioral and neural evidence for developmental disruption induced by BPA (Hajszan & Leranth, 2010; Itoh et al., 2012; Richter et al., 2007; Welshons et al., 2006; Wolstenholme et al., 2011; Golub et al., 2010). Given the coordination that must occur during this time period for correct formation of both cells in the nervous system and connections between different brain areas, it is possible that a chemical, such as BPA, would induce long-lasting changes in the brain. Further discussion centering on the previously demonstrated effects of BPA on brain and behavior is presented in the following chapters.

General Rationale and Aims for Current Studies

The following completed studies aim to clarify whether BPA exposure during gestation and early postnatal development alters cognitive behavior and anatomy of the prefrontal cortex in adulthood. In order to mimic human exposure *in utero*, pregnant rats received oral administration of various low doses of BPA (0, 4, 40, or 400ug/kg/day) throughout the entire period of gestation. Importantly, several papers have suggested that exposure of mice to ~400ug/kg/day produces 24‑hr unconjugated serum BPA

levels slightly below the levels that have been demonstrated in human women (Taylor et al., 2011, Vandenberg et al., 2010), establishing the highest dose in this study as physiologically relevant. Since rats are born at a premature state compared to humans, offspring of the dosed pregnant rats received oral administration of BPA until the 10th day after birth. Adult behavior and anatomy of the prefrontal cortex were assessed to determine whether exposure to BPA during early development induces long-lasting neural changes.

The second chapter focuses on whether administration of BPA during development induces changes in puberty onset, body weight, liver weight, brain weight, and levels of thyroxine (T4). Changes in puberty onset and body weight have been a particular concern to toxicologists given that human females are trending towards earlier onset of puberty and that obesity and body-weight associated diseases, such as diabetes, have been increasing drastically within the last few decades. Estimates suggest that these trends are likely to persist in the future. Attainment of these various endpoints allows for a more comprehensive view of the potential effects of BPA. They also add to the current available research regarding BPA, given that several other studies have examined the same endpoints.

The third chapter focuses on behavioral changes associated with BPA. Rats exposed to BPA during early development were trained in adulthood on the 17-arm radial maze with both baited and unbaited arms, a challenging task that has yet to be examined with respect to BPA. This task allows for a more detailed view of effects compared to other behavioral tasks, given that both working and reference memory errors are analyzed. Also, the increased difficulty of this task compared with other

cognitive tasks, such as the water maze, allow for better detection of small changes in behavior. Both males and females were used in this study to determine whether this potential toxicant alters sex differences, which have been previously demonstrated in this task, and/or whether BPA induces sex-specific alterations. Long-lasting behavioral changes in response to BPA may reflect changes in development of the brain, which is the focus of the fourth chapter.

Utilizing the same rats that were behaviorally tested, the third and final study examined adult neuron and glia number in the medial prefrontal cortex (mPFC), a structure that when lesioned, impairs acquisition of the radial arm maze task (Kolb et al., 1982; Kesner et al., 1987; Joel et al., 1997). White matter volume was also assessed in the area adjacent to the prefrontal cortex. Assessment of these endpoints converges with results of the behavioral study to determine whether changes in the brain are concurrent with changes in behavior.

To conclude, the following studies are designed to help clarify whether doses of BPA that correspond to human exposure and are currently considered “safe” by the EPA and FDA have long-lasting impacts on the brain and behavior. The hypothesis is that perinatal exposure to BPA would alter learning and sex differences in radial arm maze. Additionally, this endocrine disruptor may also alter normal development of, or sex differences in, the prefrontal cortex.

Chapter 2:

Effects of Bisphenol A on Growth and Development

Introduction

Several emerging concerns pertaining to BPA exposure during early development are the potential for this endocrine disruptor to contribute to changes in body weight and puberty onset. Many studies performed with rodents have demonstrated increases in body weight in response to low doses of BPA during early postnatal development (Rubin et al., 2001), at weaning age (Howdeshell et al., 1999), at times corresponding to the onset of puberty (Rubin et al., 2001; Miyawaki et al., 2007), and in adulthood (Nikaido et al., 2004; Rubin et al., 2001; Patisaul & Bateman, 2008). Interestingly, a recent review highlighted the evidence that these increases in body weight are either only visible or are more exaggerated in females than males (Rubin & Soto, 2009). However, there are also a few studies that fail to find significant changes or report decreases in body weight (often seen when using doses that exceed those expected to occur in humans) after exposure to low dose BPA (Stump et al., 2010; Nakamura et al., 2012; Newbold et al., 2007; Tyl et al., 2002; Tyl et al., 2008). These differences in the results of studies are likely due to differences in the dose of BPA, dosing duration and method, and the strain of the rat or mouse used to evaluate the effects of BPA. Given that changes in body weight have mainly been demonstrated in mice and Sprague Dawley rats, it is important to ascertain whether changes in body weight after BPA exposure are also apparent across several different doses and in different strains of rats (i.e. Long-Evans). Long-lasting changes in body weight may be

indicative of alterations in adipogenesis or metabolic processes. Substantial increases in obesity have been seen in the human population over the last few decades, and these epidemiological changes coincide with our increased use and exposure to BPA. Therefore, it is important to use controlled studies in rodents to further evaluate the relationship between body weight and BPA exposure.

Altered puberty onset in females and males is another potential effect of developmental exposure to BPA. Puberty onset in female rats, as assessed by vaginal opening, has been examined in several studies. Honma et al. (2002) reported earlier puberty onset in female mice that had been exposed to 20µg/kg/day through subcutaneous injections from gestational days 11-17, although no effects were found when rats were given 2µg/kg/day. Additionally, another study reported earlier vaginal opening in Long-Evans rats that were injected with 50µg/kg/day (but not 50mg/kg/day) from postnatal day 0 to postnatal day 3 (Adewale et al., 2009), which may be due to alterations in RFamide-related peptide-3 neurons in the hypothalamus (Losa-Ward et al., 2012). Contrary to these results, several studies have found no effects on vaginal opening at oral doses ranging from 2 to 200µg/kg/day during gestation and lactation (Tinwell et al., 2002; Ryan et al., 2010; Howdeshell et al., 1999). In addition to accelerated puberty in females demonstrated by changes in vaginal opening, it has also been reported in females that BPA during early development increased the age at which the first estrus cycle occurred (Howdeshell et al., 1999). Consequently, these mixed results suggest a potential response to BPA and a need for further studies to determine whether BPA induces earlier pubertal onset in female rodents.

Preputial separation is an external marker of puberty in male rodents. This occurs when the glans penis becomes anatomically disconnected from the prepuce, allowing for retraction of the latter (Korenbrot et al., 1977). To date, several studies have examined whether low dose BPA affects preputial separation (Tinwell et al., 2002; Kato et al., 2006; Tyl et al., 2002, 2008). From these studies which used either mice or Sprague-Dawley rats, only one that utilized an extremely high dose (100mg/kg/day) reported delayed preputial separation. Therefore, studies using other strains of rats and doses that correspond to human exposure are needed.

Collection of other measures of general growth and development, such as liver weight, aids in the determination of whether BPA is a developmental toxicant. Changes in liver weight are often used a marker for general toxicity, in that increases in liver weights may be indicative of increases in enzyme induction, hyperplasia, or hypertrophy of the liver (Amacher et al., 1998; Williams & Iatropoulos, 2002). All of these specific changes in liver anatomy and function could point to a potential mechanism of increased liver metabolism. To date, no effects on adult liver weight have been reported in the few studies that assessed this measure after early developmental exposure to BPA in doses at or below 50mg/kg/day (Tinwell et al., 2002; Tyl et al., 2002; Tyl et al., 2008).

Changes in litter size, implantation sites, and sex ratio would represent alterations to fertility in the dams and can be indicative of general reproductive function. Although several studies have reported decreased litter size and implantation sites after extremely high doses (>400mg/kg/day), no studies have reported changes due to administration of lower doses of BPA (Tyl et al., 2002; Kim et al., 2001; Ema et al.,

2001; Negishi et al., 2003; Tinwell et al., 2002). Furthermore, sex ratio, although only assessed in handful of studies, is not altered by low or high doses of BPA (Ema et al., 2001; Negishi et al., 2003; Tinwell et al., 2002).

Lastly, brain weight is a measure that is designed to give indications on whether a toxicant, such as BPA, causes global changes in the brain. However, changes in brain weight could be due to a variety of mechanisms, including but not limited to changes in gray or white matter volume, the total number of cells, or increases in synapses or dendrites. To date, no developmental BPA-induced changes in brain weight have been reported in doses ranging from 0.2-200µg/kg/day (Ferguson et al., 2011; Ema et al., 2001).

In the current study, measures of general growth and development were assessed in both the dams and offspring. Measures in the dam were collected immediately following weaning and included body weight, liver weight, brain weight, and implantation sites. In addition, litter size and sex ratio of the litter on postnatal day 1 were assessed. Offspring measurements included body weight from PND 1-9, at weaning, and in adulthood. Additionally, adult brain and liver weight, and puberty onset were also assessed. Given that the current "safe" dose is derived from data obtained in adult animals, it is important to determine whether BPA given during a proposed critical period (gestation and early postnatal development) does, in fact, differ from its effects on adults. If effects are found that are specific to early exposure to BPA, it would question the current standard of using adult animals to determine the potential toxicology of endocrine disruptors.

Methods

Breeding and Housing Procedures

Adult (>80 days old) female and male Long Evans rats were obtained from Harlan and used as breeders. Due to the long length of time needed to behaviorally test the exposed offspring, two separate cohorts of animals were used. Numbers of litters for each treatment are as follows: oil =9, 4µg/kg/day=10, 40µg/kg=13, 400µg/kg/day=12. Therefore the sample size was 9-13 litters/treatment group. Precautions were undertaken to minimize exposure to materials that might contain BPA (recycled paper, polycarbonate plastics, etc.). All rats in the current study were housed in clear, polysulfone cages (except during breeding) and given access to reverse osmosis water in glass bottles. The diet used in this experiment (Harlan 2020X) contained low, but stable amounts of phytoestrogens. Prior to breeding, female breeders were pair- or triple-housed, while males were single-housed due to aggressive behavior towards cagemates displayed in the first several days after arrival of the 1st cohort. Breeding procedures did not begin until at least 2 weeks after arrival. During the time between arrival and breeding, male and female breeders were handled at least 3 times, while females received 100µl of tocopherol-stripped corn oil pipetted onto a ½ of a cookie (Newman's Own Arrowroot flavor) after each bout of handling. For breeding, one male and one female were placed in metal breeding cages and were monitored daily for the presence of a one sperm plug underneath the corresponding cage. If no sperm plug was detected after 6 nights, a different male was placed with the female for an additional 6 nights. Upon detection of a sperm plug, females were removed from breeding cages and were single-housed in polysulfone cages throughout

the remainder of the experiment. Dosing of pregnant dams was initiated on the first day of detection of at least one sperm plug.

Dam Dosing

During each day of dosing, females were weighed and solutions were mixed thoroughly by the use of a stir bar to ensure homogeneity. BPA powder was suspended in tocopherol stripped corn oil to make different solutions (0, 0.01. 0.1, or 1 mg BPA/ml oil) and these solutions corresponded to daily doses of 0, 4, 40, or 400µg BPA/kg of body weight/day throughout the entire period of pregnancy. Fresh solutions were prepared prior to each cohort of rats. 0.4µl of solution/g of body weight was pipetted on a ½ of a cookie (Newman's Own Arrowroot flavor) and was allowed to dry for several minutes before administration to each dam in their home cages. Dams consumed the ½ of a cookie within 5 minutes.

Pup Dosing

The day of parturition was designated as postnatal day (PND) 0. Similar to dosing of the dams, solutions (0, 0.002, 0.02, and 0.2mg BPA/ml of oil) were mixed daily and prepared prior to the start of each cohort. Direct oral administration of the same doses of BPA solutions that were given to dams (0, 4, 40,and 400µg BPA/kg of body weight/day) were given to pups beginning the day after birth (PND 1) and continued until PND 9. Although many previous studies relied on lactational transfer of BPA from dams to pups as a route of exposure, there has been recent evidence to suggest that using this method results in extremely low exposures to the pups (Doerge et al., 2010c). Procedures for this portion of the experiment included placing the dam in a separate cage and recording weight and sex for each individual pup. Pups were then

picked up, a pipette was placed in the mouth, and the solution was dispensed. Pups were given 2 separate bouts of oral dosing for a total volume of 2µl solution/g body weight. Body weights were recorded for all offspring at this time, and therefore, results for average body weight during early development (PND1-9) were determined by calculating the average body weight for females and males separately from each litter. Litter size and sex ratio was assessed on PND 1. At PND 2, litters were culled to a maximum of 10 pups, while trying to maintain a balanced sex ratio in each litter as much as possible.

At PND 23, all offspring were weaned and 2 females and 2 males from each litter were weighed and retained for future behavioral and neuroanatomical endpoints. Dams and remaining littermates were sacrificed at this time, and various measurements were recorded, including body weight, liver weight, brain weight, and implantation sites in the dams. Analyses of offspring body weight at weaning represent an average of all of the females and all of the males from each litter. All rats retained for later measures were double-housed with same sex littermates and handled once a week for the remainder of the experiment.

Beginning at PND 25, females were checked daily for vaginal opening, and males were visually checked daily for preputial separation beginning on PND 30 to determine onset of puberty. Puberty onset was averaged from the 2 female and 2 male offspring from each litter. Prior to food restriction for behavior in adulthood, weights for all rats were recorded around PND 85 and these weights were used for analysis of adult body weights. Ad libitum food was returned immediately upon completion of behavioral testing. Although body weights were also measured prior to sacrifice (PND 140) for the

later experiments, these were not analyzed due the variability in time (2-5 weeks) between the end of food deprivation used for behavioral training and sacrifice. At the time of sacrifice (around PND 140), liver and brain weights of the offspring were also recorded. These measures taken in adulthood from the offspring (adult body, brain, and liver weights) were calculated as an average of the two offspring from each sex that were available from each litter.

T4 Serum Analyses

At weaning, trunk blood was collected from littermates that were not used for further behavioral and anatomical endpoints. Serum from a maximum of one female and one male from each litter was analyzed for thyroxine levels (T4). Serum T4 was measured by radioimmunoassay based on previously reported methods (Schneider et al., 2006; Taylor et al., 2008) from a maximum of one male and one female from each litter. 5 µl of serum was assayed per tube and added to 200µl of GAB. The primary antibody used was a polyclonal rabbit anti-T4 antibody (Cat#20-TR40, Fitzgerald Industries International, Concord, MA). Approximately 0.006 µCi of [125I]-T4 was added on the third day. On the fifth day, 50µl of 200µg/ml (10µg) solution of rabbit immunoglobulin was added, followed by 100µl of a GAR secondary antibody solution (Cat#R0881, Sigma) prepared at 60% of the manufacturers recommended volume for a final dilution of approximately 1:8. Tubes were incubated at room temperature for 30 minutes before addition of 1ml of a 25% wt/vol solution of PBS/PEG. Tubes were then centrifuged, aspirated, and counted in a gamma counter. All samples were run single assay. The serum T4 assay had lower and upper limits of detection of 0.05µg/dl and 10.5µg/dl, respectively for a 5µl sample. The sensitivity of the assay as determined by 3

standard deviations from the zero standard and 3 standard deviations from the nonspecific binding level. The coefficient of variation was 8.1%.

Statistical Analyses

Measures taken from the dams were analyzed with ANOVAs (SPSS; version 19; SPSS Inc., Chicago, IL) with treatment as the independent factor and cohort as a covariate. Statistical significance was set at $p<0.05$.

All measures in the exposed offspring were analyzed using ANOVAS with the between-subject variable being treatment and using cohort as a covariate. One way ANOVAS were used as posthoc tests in order to include cohort as a covariate. In cases where data was available from more than one littermate per sex, averages were calculated for each sex of each litter. Due to large, well-known sex differences in most of the measures, females and males were analyzed separately. In order to account for body weight affecting liver weight, liver weight was divided by body weight measured at the time the animals were sacrificed. Statistical significance was set at $p<0.05$.

Results

Maternal Measures

Adult female rats that were exposed to BPA during their pregnancy did not exhibit any significant differences in body weight, brain weight, liver weight, or number of implantation sites (Table 1). Litter size and sex ratio of the offspring were not significantly affected by BPA treatment (Table 1). Sample sizes for these measures were as follows: 0µg/kg = 9, 4µg/kg = 10, 40µg/kg =13, 400µg/kg =12. The remaining results are those obtained from the offspring of these dams.

Offspring Measures

Results for effects of BPA on body weight at early postnatal development, at weaning, and in adulthood (PND 85) are shown in Figure 1. BPA treatment did not affect body weight in males or females at any age or in any of the doses examined. Additionally, no significant treatment effects in males or females were found in puberty onset, adult brain weights, or adult relative liver weights (Table 2). Sample sizes for these measures were as follows: 0µg/kg = 9, 4µg/kg = 10, 40µg/kg =13, 400µg/kg =12.

Results for thyroxine levels (T4) are shown in Figure 2. Sample sizes (shown in the graphs) for females were 0µg/kg = 8, 4µg/kg = 9, 40µg/kg =11, 400µg/kg =12 and for males were 0µg/kg = 9, 4µg/kg = 9, 40µg/kg =11, 400µg/kg =10. There was a significant effect of treatment in T4 levels at weaning in females ($p<.05$) and a nonsignificant trend in males ($p=.09$). Analysis of T4 levels between the BPA-treated and control animals showed a significant increase in T4 levels in female rats exposed to 4µg/kg/day compared to both the control group ($p<.05$) and the group that received 40µg/kg/day ($p<.01$). There was also a strong trend in females towards lower levels of T4 in the 40µg/kg/day treatment group compared to the 400µg/kg/day group ($p=.06$). In males, the 4µg/kg/day group had lower levels of serum T4 than the 40µg/kg/day group ($p<.05$). These results suggest effects of BPA on T4 levels at weaning represent a non-monotonic dose response curve.

Discussion

The current study found no significant effects of BPA on maternal endpoints or in litter size or sex ratio of the litter. The lack of effects on maternal body weight, litter size, and sex ratio are consistent with past studies that used comparable doses of BPA (Tinwell et al., 2002; Tyl et al., 2002, 2008; Ema et al., 2001; Somm et al., 2009,

Ferguson et al., 2011; Negishi et al., 2003). Therefore, BPA given during pregnancy does not result in changes in general toxicological measures in dams assessed several weeks after discontinuation of treatment.

BPA exposure during early development did not alter body weight, brain weight, liver weight, or puberty onset in males and females. The lack of effect on body weight is in contrast with many studies that have demonstrated significant increases in body weight after exposure to BPA, especially in females (Howdeshell et al., 1999; Nikaido et al., 2004; Rubin et al., 2001; Patisaul & Bateman, 2008; Miyawaki et al., 2007; Somm et al., 2009). However, there is also support for a lack of effects on body weight. For example, other studies have demonstrated either decreases or no significant changes in body weight in rodents exposed to BPA during early development (Stump et al., 2010; Nakamura et al., 2012; Newbold et al., 2007; Tyl et al., 2002; Tyl et al., 2008; Kobayashi et al., 2002). These differential effects in the response to BPA may be mediated by methodological differences between the studies such as diet and the dose of BPA that was being tested. There is also evidence that position of the fetus in the uterus during gestation may mediate some of these differences given that females surrounded by 2 males did not show weight differences at weaning, while females surrounded by 0 or 1 male exhibited increases (Howdeshell et al., 1999).

Puberty onset was not significantly affected in either males or females rats perinatally exposed to BPA. In previous studies that investigate effects of BPA at doses below 500µg/kg/day, accelerated vaginal opening in females has been reported (Honma et al., 2002; Adewale et al., 2009), although there have been several studies that fail to find effects on this same measure (Ryan et al., 2010; Tinwell et al., 2002). The only

studies that examined preputial separation after exposure to BPA, at doses comparable to the current study, did not find effects on this measure (Ema et al., 2001; Tyl et al., 2002; Kato et al., 2006; Tyl et al., 2008), which is consistent with the lack of effects exhibited in the current study. No effects on relative liver or brain weight were apparent in rats that were perinatally exposed to bisphenol A. These results are consistent with past studies that have examined these measures (Tyl et al., 2002; Kobayashi et al., 2002).

Despite a lack of changes in most of the measures that are often used to determine whether chemicals are potential toxicants, there were significant alterations to T4 at weaning. Importantly, these changes occur are apparent several weeks after the discontinuation of treatment. Given that BPA has a short half life (Völkel et al., 2002; 2005), it is highly unlikely that BPA is still present in the blood of the offspring, suggesting that BPA exposure induced lasting changes in hormone levels. The finding that BPA may alter the thyroid hormone system is not without precedent. For example, BPA antagonizes thyroid hormone receptors and increases expression of thyroid responsive genes (Moriyama et al., 2002; Zoeller et al., 2005). Additionally, maternal exposure to 1mg/kg, 10mg/kg and 50mg/kg BPA increased levels of thyroxine in both males and females at PND 15, but not at PND 4, PND 8, or PND 35 (Zoeller et al., 2005). However, the doses used in this previous study are unlikely to correspond to doses of BPA that occur in the human populations. BPA given during gestation and early postnatal development also resulted in a transient hyperthyroidism (at postnatal day 7) in a lower dose of BPA and hypothyroidism (at postnatal day 21) in a higher dose of BPA in male rat offspring, while no effects were seen in female offspring (Xu et al.,

2007). How the doses in Xu et al. (2007) correspond to those used in the current study are unknown, given that the BPA administration was given in the water (in mg/L) and the amount of water that the dams consumed was not reported. In conclusion, T4 levels at weaning are significantly altered after early exposure to BPA. More studies are needed to determine the immediate effects of BPA during dosing and whether changes in T4 reported here persist into adulthood.

To summarize, the results obtained in this study suggest that developmental BPA, at levels corresponding to estimated exposures in humans, does not induce alterations in measures assessed in the dams. However there were significant alterations to thyroxine levels at weaning, suggesting that current methods (such as measurement of body weight) often used to screen for potential toxicants are inadequate. The results obtained here do support the idea that BPA can induce alterations to the thyroid hormone system. While there is a substantial amount of evidence that alterations the thyroid hormones during the prenatal period are important for neuron, glia, and white matter development (Sharlin et al, 2008; Leonard et al., 2008; Bernal, 2005. Morreale de Escobar et al., 2004; Berbel et al., 2010), there are no studies to date that assess whether T4 alterations at weaning age impact neural structure in adulthood. This study did not collect serum during the time of BPA dosing, so it is unknown whether similar changes are seen at this time when the amount and magnitude of neural changes are large.

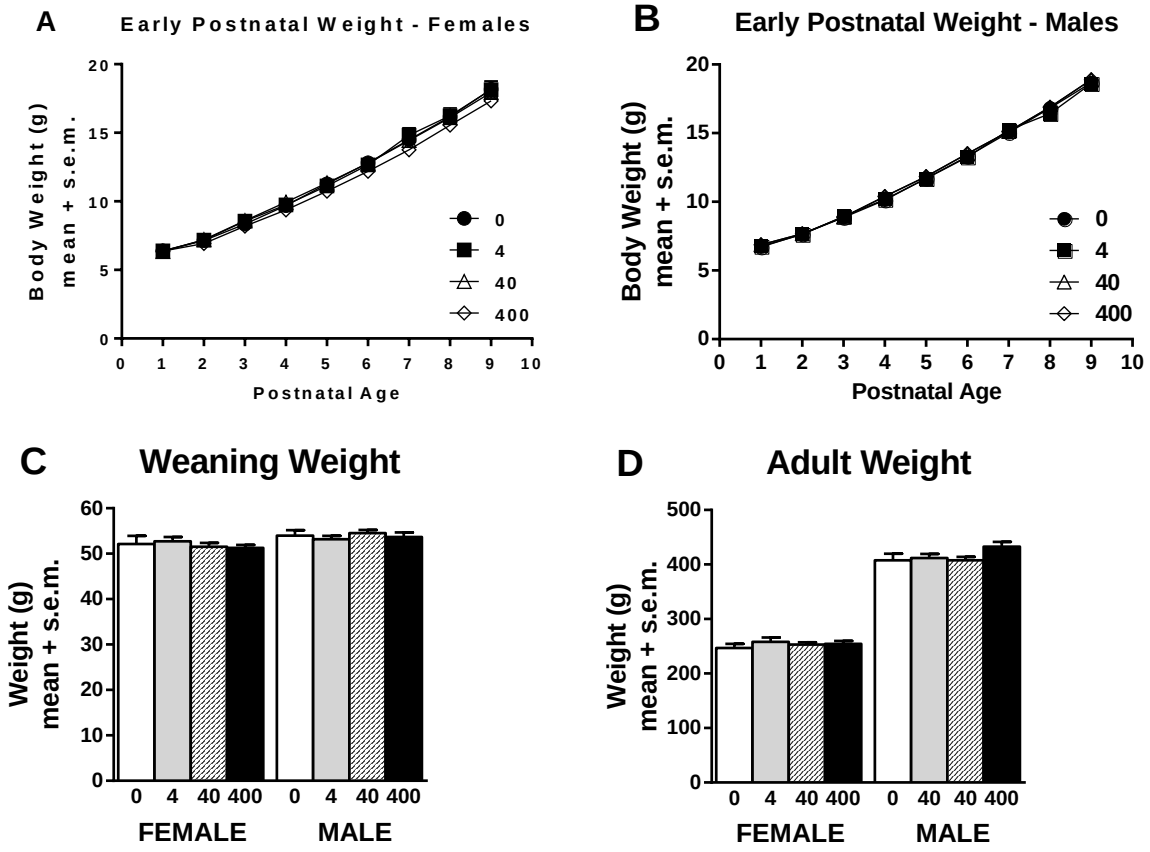
Figures and Tables

Table 1. Maternal Measures

Dose (µg/kg)	Body Weight (g)	Brain Weight (g)	Relative Liver Weight (Liver Weight/BW)	No. of Implantation Sites	Litter Size	Sex Ratio (#males/ #females)
0	281.1±7.3	1.93±0.03	0.0594±0.0030	12.2±0.9	10.8±0.9	1.05±0.22
4	279.7±3.7	1.85±0.04	0.0587±0.0019	12.7±0.4	11.4±0.7	1.06±0.19
40	281.6±5.2	1.91±0.02	0.0582±0.0009	12.7±0.6	11.8±0.7	0.77±0.10
400	279.0±4.8	1.91±0.02	0.0617±0.0014	13.0±0.6	12.0±0.6	0.91±0.12

No significant differences were seen in maternal body weight, brain weight, relative liver weight, the number of implantation sites, litter size, or sex ratio.

Figure 1. Offspring Body Weight



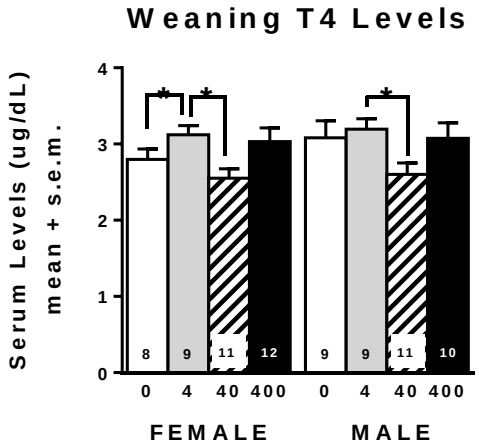
BPA did not alter body weight in males or females during early postnatal development, weaning, or in adulthood.

Table 2. Offspring Measures

Dose (µg/kg)	Sex	Puberty Onset (PND)	Relative Liver Weight (Liver Weight/BW)	Brain Weight (g)
0	Female	33.2±1.0	0.0330±0.0004	1.85±0.02
4	Female	32.7±1.0	0.0323±0.0007	1.84±0.01
40	Female	31.5±0.6	0.0321±0.0005	1.84±0.01
400	Female	32.5±0.8	0.0323±0.0007	1.84±0.03
0	Male	43.2±0.6	0.0332±0.0007	1.99±0.03
4	Male	42.6±0.4	0.0327±0.0007	1.97±0.02
40	Male	42.5±0.3	0.0333±0.0005	1.95±0.02
400	Male	42.9±0.4	0.0338±0.0006	1.99±0.02

BPA did not alter puberty onset, adult relative liver weights, or brain weights in males or females.

Figure 2. Thyroxine Levels



A significant treatment effect in females and a nonsignificant trend towards a treatment effect in males (p=.09) revealed a nonmonotonic dose response curve with the moderate dose showing a significant decrease in T4 levels at weaning.*p<.05

Chapter 3:

Effects of Perinatal Bisphenol A on Radial Arm Maze Behavior

Introduction

Particular concern has arisen regarding the potential for perinatal exposure to BPA to exert long-lasting changes in behavior. The National Toxicology Program (NTP) (2008) reviewed published studies to conclude that there are some concerns for adverse effects of developmental exposure of BPA to fetuses, infants, and children on the brain and behavior. However, the available investigations that examine long-lasting changes in behavior in both males and females are limited. The majority of animal studies investigating behavioral changes in response to perinatal exposure to BPA have focused on anxiety-related behaviors and novelty seeking. Low dose perinatal BPA increases anxiety as evident by a reduction in entries or time spent in open arms in the elevated plus maze (Patisaul & Bateman, 2008; Cox et al., 2010; Ryan & Vandenbergh, 2006) or by a decreased time spent in a novel area in a novel preference test (Adriani et al., 2003). Several additional studies using the elevated plus maze or open field task reported a loss of sex differences (Gioiosa et al., 2007; Rubin et al., 2006; Fujimoto et al., 2006; Kubo et al., 2001; 2003). In Gioiosa et al. (2007), control females spent more time in the center area of an open field than control males. However, male mice exposed to 10µg/kg/day BPA significantly increased their time in the center in comparison to male controls and females exposed to the same dose of BPA slightly decreased their time in the center. Similar results, i.e. abolishment of sex differences, were found when these mice were tested in an elevated plus maze and a novelty task.

Lastly, increases in basal levels of corticosterone in females and increases in levels after exposure to a novel environment (Poimenova et al., 2010) support the idea that perinatal BPA results in long-lasting changes in anxiety and anxiety-related behavior that may be mediated by changes in stress hormones.

Long-term effects of perinatal BPA on social behavior are not as extensive or definitive as those demonstrated in tasks that assess anxiety. 40µg/kg/day and 400µg/kg/day BPA given during early development affected different aspects of play behavior in a dose dependent manner with some behaviors, such as play between females being increased and other aspects of social behavior, such as sociosexual interest, being decreased (Dessi-Fulgheri et al., 2002). However, in a related study in which female rats were exposed to 40µg/kg/day, play behavior directed towards males decreased, while there was no change in play directed towards other females (Porrini et al., 2005). These findings were observed at ages that correspond to adolescence. Consequently, it is unknown whether changes in social behavior persist into adulthood.

Lastly, and most relevant to the studies proposed here, are changes to learning and memory after exposure to low doses of BPA during the perinatal period. Several studies have employed the water maze to determine whether perinatal exposure to BPA alters spatial learning and memory (Carr et al., 2003; Xu et al., 2010; Goncalves et al., 2010; Nakamura et al., 2012). In this task, rats are trained over the course of several days to find a hidden platform using spatial cues located in the training room. On a probe trial, the platform is removed and different measures are utilized to determine memory retention. Although a number of studies mentioned above have used the water maze, differences in methodology and observed effects make it difficult to extrapolate

whether BPA has long-lasting effects on behavior in this task. Carr et al., (2003) administered either 100µg/kg/day or 250µg/kg/day of BPA to males and females from postnatal days 1-14 and rats were trained and then tested at ages corresponding to adolescence (from PND 33-40). During the acquisition phase of this task, control males and females exhibited a sex difference in the slope of performance in terms of latency, while rats exposed to 100ug/kg/day did not. However, no significant differences between treatments were seen during acquisition. Females treated with the larger dose of BPA also showed an increased amount of time spent in the target quadrant on a probe trial, while neither dose of BPA had an effect on male behavior.

While this study (Carr et al., 2003) stated conclusions that BPA alters performance in the water maze, there are important methodological considerations that make interpretation of these results difficult. For instance, the age at testing corresponds to an approximate time when females are exhibiting or already have exhibited physical onset of puberty. In contrast, males were unlikely to have exhibited external markers of puberty at this age. Therefore, these differences in the status of puberty may be contributing to the differences in effects observed between the two sexes. This study only reported latency measures during acquisition and it was not tested whether BPA impacted locomotor activity, such as swim velocity. For these reasons, path length is a better measure of learning in this task. Also, significant differences between treatments in the probe trial without differences in acquisition make interpretation difficult, as these results could be indicative of perseverative behavior. Lastly, it is not known from this particular study whether these alterations would persist

into adulthood. Therefore, the following discussion of other studies examining the water maze will focus on behavior in adulthood.

Several other studies have utilized the water maze to examine the potential of early exposure to BPA to disrupt cognitive behavior in adulthood. Goncalves et al., (2010) did report some differences in performance in the water maze in male and female rats. Exposure to 40µg/kg/day resulted in enhancements or impairments between different treatment groups in terms of latency and path length during the training days in this study (Goncalves et al., 2010) depending on whether exposure occurred during gestation, during lactation, or during both developmental time points. However, due to the fact that rats showed differences in swim velocity which are indicatively of potential disruptions to motor ability, only path length (and not latency to find a platform) are valid measurements. Impairments were evident during training days 2 and 4 between rats exposed to BPA during lactation only (in terms of path length) and during the probe trial in rats exposed during gestation. Another study examining performance in the water maze did find impairments in the higher dose groups (50mg/kg, 5mg/kg, .5mg/kg), but not the lowest dose group (.05mg/kg) during learning and impairments for memory of the task on the probe trial in the 50mg/kg and .5 mg/kg treatments group compared to controls (Xu et al., 2010). In contrast, another study failed to find significant effects on water maze performance after subcutaneous injections of 20µg/kg/day during gestation and lactation to the dams (Nakamura et al., 2011).

Only a few tasks other than the water maze have been employed to determine potential effects of BPA on learning and memory. Two of the previously mentioned

studies reported impairments in the passive avoidance task in adult males and females (Goncalves et al., 2010; Xu et al., 2010). Kubo et al. (2001) found that 1.5mg/kg/day abolished a sex difference in a passive avoidance task, although this dose is unlikely to mimic human exposures.

Although these studies may suggest that bisphenol A may alter water maze and passive avoidance performance, there are important considerations with respect to task differences. The water maze utilizes the fact that rats find the water aversive, and it has been shown that exposure to water at a temperature range used in the previously discussed studies (around 25ºC) results in an increase in serum corticosterone levels (often used as an indicator of stress) of two fold compared to rats not exposed to the water (Sandi et al., 1997). Given the available and consistent demonstrations of changes in anxiety behavior after BPA (Patisaul & Bateman, 2008; Cox et al., 2010; Ryan & Vandenbergh, 2006; Adriani et al., 2003; Gioiosa et al., 2007; Rubin et al., 2006; Fujimoto et al., 2006; Kubo et al., 2001; 2003), water-induced changes in anxiety and stress may confound results found with the water maze behavior. Additionally, similar concerns arise when reviewing studies in which passive avoidance behavior is altered after early exposure to BPA (Goncalves et al., 2010; Kubo et al., 001; Xu et al., 2010). This task also uses an aversive stimulus (footshock) to induce motivation to perform the task. Therefore, it is important to assess behavior in appetitive tasks to avoid these potential confounds.

Only one study has utilized dry maze tasks to assess effects of BPA on learning and memory. In Ryan and Vanderberg (2006), female mice were exposed to 0, 2, or 200µg/kg/day BPA during gestation and lactation. Mice were then tested on the Barnes

maze around PND 42 and the radial arm maze about 1 week later. No treatment effects of BPA were seen in either task. Also, a lack of learning in the controls in the radial arm maze in this study suggests that motivation/reward to solve the maze was not sufficient or that not enough trials were given to show learning over time, making it difficult to draw conclusions from this study.

Given the available evidence, it is possible that BPA alters behavior or sex differences in behavior. However, it is obvious that more studies are needed to identify dose-dependent effects of perinatal exposure to BPA, especially with respect to cognitive-related behaviors. Many of the studies discussed in the previous paragraphs failed to utilize a wide range of doses of BPA relevant to human exposure and/or only used either males or females. Furthermore, several of the behavioral studies did not tightly control for BPA exposure from other sources, such as water, food, or cages. Controlling for outside BPA exposures and using several doses of BPA, the current study examined the effects of early developmental exposure to BPA on acquisition of a 17-arm radial maze task. Several reasons exist for why this behavioral task was implemented. First, a 17-arm radial maze task was utilized since it is a task that is considered challenging, making it more likely to discern smaller effects of chemical exposures. Second, previous results from our lab and others have demonstrated sex differences in performance on this radial arm maze task, with males committing fewer reference and working memory errors than females (Seymoure et al., 1996; La Buda et al., 2002; Beatty, 1984; Mishima et al., 1986; Tees et al., 1981; Williams & Meck 1990). The current experiment utilized several doses of BPA to determine effects of perinatal exposure on radial arm maze behavior in adult males and females.

Methods

Radial Arm Maze

Starting in adulthood (PND 85), two female and two male rats from each litter of those described in Chapter 2 were weighed and placed on food restriction. During this time, rats were daily given 3 pellets of the food reward that was used during behavioral training. The target weight was 85-90% ad libitum and due to sex-specific increases in body weight during this age, the target weight was increased by 5 grams weekly for males only. Only 1 female and 1 male from each litter were tested in the radial arm maze. Habituation began 5 days after the start of food deprivation and consisted of placing rats in the center platform of a 17-arm radial maze. One pellet of food reward was placed in each arm in a random location and 2 pellets where placed in the center platform. Rats were allowed to run freely for 12 minutes for 4 consecutive days to acclimate rats to training procedures.

Starting on the day after completion of habituation, rats were trained for 6 days/week for 4 weeks. Prior to training, one food reward pellet was placed at the end of 11 out of the 17 arms. The remaining arms did not contain a food reward. The arms that were baited and unbaited were consistent in their spatial location throughout the entire training period, so that rats had to remember not only which arms they had already visited within a day, but also the arms that never contained food reward. Once a day, rats were placed in the center of the maze and allowed to explore freely until all food rewards were eaten or for a maximum of 15 minutes. An entry into an arm was recorded when all four limbs were inside an arm. Reentry into a previously baited arm was termed a working memory error, while entry into an arm that never contained food

rewards was termed a reference memory error. After training, rats were placed back into their home cage with their untrained littermate, and the maze was cleaned with a 10% ethanol solution prior to training of the next rat. The arms were rotated 2 positions every 4 days to reduce the possibility that remaining odor cues could be used to learn this task.

Statistical Analysis

Analyses of reference and working memory errors were performed using repeated measures ANOVAS that included all of the treatments. Cohort was used as a covariate in all of the comparisons due to significant differences between the two cohorts ($p<.05$) in most measures. Due to the expectation that males and females may exhibit different responses to BPA, female and male scores were analyzed separately when assessing potential effects of treatment. In cases where indications for treatment by block interactions occurred, posthoc ANOVAs using treatment as an independent factor and cohort as a covariate compared scores of different treatment groups at individual blocks. Repeated measures ANOVAs with sex as the independent factor and cohort as a covariate were also performed between male and female controls to determine whether sex differences were apparent. Statistical significance was set at $p<0.05$.

Additional analyses were performed with all treatment groups to compare radial arm maze performance in both sexes. Analyses of working and reference memory errors were analyzed using repeated measures ANOVAS, with the between-subject variable being treatment. Litter was used as the unit of variance and sex was nested within litter. Cohort was used as a covariate in these analyses.

Results

Reference Memory Errors

All behavioral graphs depict averages for each block (6 days) of training. Reference memory errors are displayed in Fig 3. There was no significant treatment effect or interaction of treatment x block in females. However, there was a significant effect of block ($p<.05$) in females. An analysis of male reference errors revealed a weak trend towards a treatment x block interaction ($p=.08$) and a significant effect of block ($p<.01$). Comparisons at each block between the different treatment groups showed several minor indications for alterations in errors in males. In block 2, there were trends towards decreased errors in the 40µg/kg compared to the control group ($p=.09$) or the 4µg/kg group ($p=.07$). In block 4, the males that received 40µg/kg BPA had significant increases in errors compared to controls ($p<.05$), while the 4µg/kg ($p=.07$) and 400µg/kg ($p=.08$) also exhibited trends towards increased errors compared to controls. No significant sex differences were seen between the female and male control groups. When comparing all treatment groups and both sexes, there were no significant effects of treatment or sex. Samples sizes for each treatment and for each sex were as follows: 0µg/kg = 9, 4µg/kg = 10, 40µg/kg = 13, 400µg/kg = 12.

Working Memory Errors

Working memory errors are shown in Fig 4. No significant treatment, treatment x block, or block effects were observed in males or females. No significant sex differences were seen between the female and male control groups. However, when comparing all treatment groups with both sexes included in the analysis, there was a

significant effect of sex ($p<.05$). Samples sizes are the same for working memory errors as were reported for reference memory errors.

Discussion

The current study is the first to measure whether perinatal exposure, at a time that is meant to mimic human exposure, affects both reference and working memory in adult rodents, as assessed through training on the 17-arm radial maze. The results indicated only a modest effect of BPA treatment in males in reference memory errors with indications for differences occurring mostly during the last week of training. However, the only significant difference was for slight increases in reference memory errors in block 4 in the 40ug/kg group (with an average of approximately 2 more errors) compared to controls. Although the results of this study suggest that slight impairments in learning in males may result from BPA exposure, it is apparent that large changes in radial arm maze behavior are not induced.

Only one previous study examined the potential for BPA to impact radial arm maze behavior, and this study examined female mice and examined performance in an 8-arm version of the task (Ryan and Vandenbergh, 2006). This study exposed the dams to 2 or 200µg/kg/day through oral administration by gavage to the dams from gestation until the offspring were weaned. Additionally, mice were ovariectomized around PND 28, and testing on the RAM behavior occurred around PND 45. Even with the methodological differences between Ryan and Vandenbergh (2006) and the current study, such as age of testing, species, dose, and surgical procedures, a similar lack of large effects were found. The current study suggests that although some changes in

learning behavior may occur in males, these differences are small in magnitude and are unlikely to contribute to drastic changes to cognitive learning.

This cognitive task was originally chosen due the fact that a large number of arms (compared to the 8-arm radial maze) would make this task difficult and more likely to see small differences. If a task is too easy and is learned quickly, it would make it difficult to see small impairments in learning. On the other hand, if a task is too challenging, detecting small learning enhancements would be difficult. However, there was a significant effect of block in both males and females in reference memory errors with performance improving across the weeks of training. Therefore, it would be possible to see both enhancements and impairments in this measure. Contrary to reference memory errors, no significant effects of block were seen in either sex in working errors. Although the graph does seem to show that performance in this measure improves over time, the large variability in errors may contribute to inability to detect significant improvement over time.

No positive controls were used in this study because the mechanism in which BPA could alter performance on this task is unknown. Radial arm maze performance in the 8-arm version of this task has been shown to be responsive to manipulations to a wide variety of hormones, such as estrogen, testosterone, and corticosterone (Davis et al., 2005; Sprizter et al., 2011; Roskoden et al., 2005). Also, environmental factors, such as environmental enrichment, can alter performance 17-arm radial maze (Seymoure et al., 1996), indicating that this task is both appropriate and useful in determining effects of potential endocrine disruptors on cognitive behavior.

Contrary to previous reports from our laboratory in this task (Seymoure et al., 1996) and in other radial arm maze tasks (La Buda et al., 2002; Beatty, 1984; Mishima et al., 1986; Tees et al., 1981; Williams & Meck 1990), no sex differences were seen between male and female controls in either reference or working memory errors. However, when comparing all treatment groups with both sexes included in the analysis, there was a significant effect of sex in working errors. This suggests that it may have been possible to detect sex differences in controls in this measure if more rats had been added to the control group.

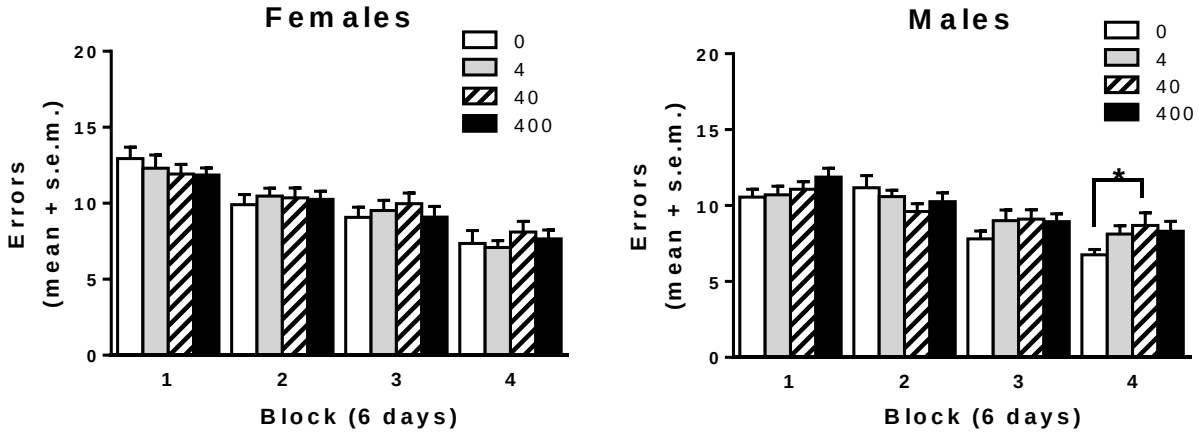
There may be several reasons for why no sex differences were observed when comparing the male and female control groups as has been reported in a previous study using the 17-arm radial maze (Seymoure et al., 1996). This discrepancy may be due to handling of both the dam during gestation and pups during early postnatal development that occurred in this study and not in Seymoure et al. (1996). Although no studies have directly tested for sex-dependent responses to early handling on radial arm maze behavior, there is evidence that handling during the early postnatal development can affect stress-related measures in males and females differently. For example, females failed to adapt to chronic stress as assessed by immobility time in a forced swim task, while males exhibited no changes in this measure after chronic stress (Papaioannou et al., 2002). Males also show exaggerated adrenocorticotropin hormone (ACTH) levels in response to shock after being handling during the first 10 days of postnatal development, while the response in female ACTH levels are unaffected by early handling (Erskine et al., 1975). Additionally, the responses to early postnatal handling on corpus callosum area (Berrebi et al., 1988) and turnover of serotonin in prefrontal

cortex (Duchesne et al., 2009) are also dependent on sex. Another potential factor that could disrupt sex differences in this study is the administration of oil during early postnatal development. Pups are not normally exposed to oil during this developmental period and it is currently unknown as to what changes this early manipulation might induce in adult behavior.

In conclusion, early exposure to BPA only leads to slight impairments in reference memory in males mostly late in training in the 17-arm radial maze task. Behavior in the radial arm maze in females is not altered by exposure to BPA. However, changes in structure of the brain after early exposure to BPA are still important to measure given the past demonstrated alterations to anxiety and stress-related behaviors.

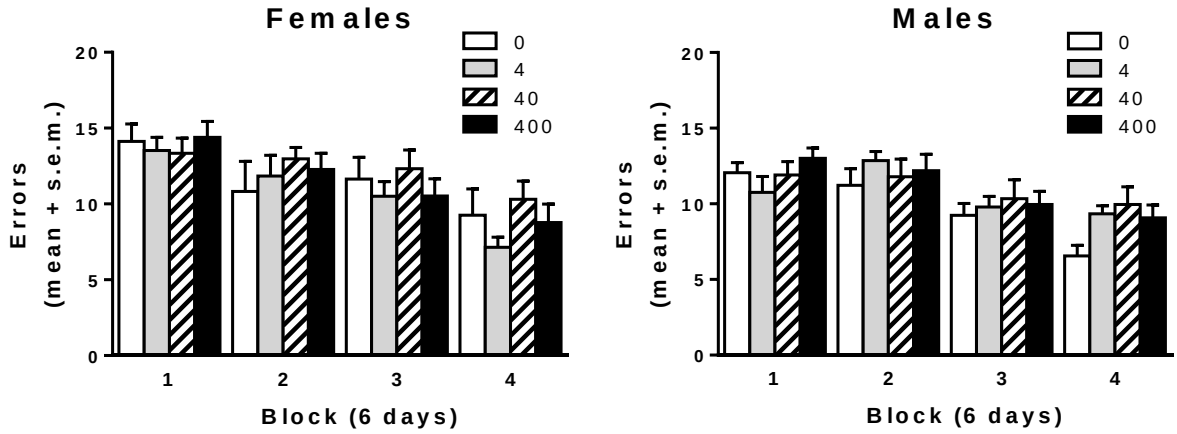
Figures

Figure 3. Reference Memory Errors



BPA did not alter reference memory errors in females. There was a trend towards a treatment x block interaction in males (p=.08). The only significant difference was between 0 and 40 in block 4.*p<.05

Figure 4. Working Memory Errors



BPA did not alter working memory errors in males or females.

Chapter 4:

Effects of Perinatal Bisphenol A on Anatomy of the Prefrontal Cortex

Introduction

To date, evidence suggests that BPA exposure during early development has the ability to alter structure of the brain. One must consider the magnitude and variety of changes, including proliferation, migration, and differentiation that the brain must undergo to reach the level of maturation that is observed after gestation and early development. The dynamic state of this system coupled with the large number of processes that must occur in a time and region-specific manner make this age particularly susceptible to perturbations by potential toxicants. In addition to BPA having actions at both estrogen and thyroid receptors (two hormones that are involved in the development of neural systems), it also possesses the ability to cross the blood-brain barrier (Sun et al., 2002). Consequently, it is imperative to determine whether endocrine disruptors, such as BPA, alter neuroanatomical development of the brain.

One way in which developmental exposure to BPA could induce changes in neuroanatomy is by altering neuron number. Several studies investigating neuron number in different areas of the brain in both males and females have demonstrated alterations in adult sex differences after exposure to BPA. For instance, sex differences in neuron number and volume of the locus coeruleus were completely reversed when rats were exposed to 30µg/kg/day, 300µg/kg/day, or 1.5mg/kg/day BPA during early development (Kubo et al., 2001; 2003). Additionally, BPA administration abolished sex differences in corticotropin-releasing hormone (CRH)-immunoreactive neurons in the

bed nucleus of the stria terminalis (Funabashi et al., 2004). This effect was due to a BPA-mediated increase in neurons in males and a decrease in females. In the same study (Funabashi et al., 2004), CRH neuron number in the sexually dimorphic nucleus of the preoptic area of the hypothalamus was not changed in males or females, suggesting that changes in sex differences due to BPA are brain-area specific. Two studies have demonstrated that BPA changes neuron number in the anterior periventicular nucleus of the hypothalamus (AVPV), an area important for regulation of reproductive functions. BPA decreased tyrosine hydroxylase-positive neuron number in females, while having no effect in males, resulting in abolishment of sex differences exhibited in controls (Rubin et al., 2006). In this study, only the anterior subregion of this nucleus was affected by BPA treatment, again lending support to the idea that BPA effects are likely confined to particular regions of the brain. Additionally, the number of oxytocin-immunoreactive neurons in the AVPV was increased in adult female rats after early developmental exposure to 50µg/kg/day and 50mg/kg/day of BPA (Adewale et al., 2011). Males were not examined in this study, so it is unknown whether there are sex differences in this response. Interestingly, all of the above mentioned studies that found changes in sex differences in the brain after BPA exposure were limited to regions where females have more neurons than males. These studies mentioned above indicate that some sex differences in neurons are altered by BPA. However, more studies that investigate changes in neurons in both males and females and in different areas of the brain, such as those involved in mediating cognitive function, are necessary.

Although it is very likely that BPA may contribute to changes in neuron number in a variety of different brain areas, virtually no evidence is available to determine whether similar effects are seen in areas associated with learning and memory behavior, such as the hippocampus and prefrontal cortex. An approximate dose of 70µg/kg/day BPA given during gestation and lactation decreased the density of NeuN-positive neurons in the hippocampus at PND 20 in males and females (Kunz et al., 2011). However, conclusions that can be drawn from this study are very limited due problems associated with studies that only report density measures. Assessment of neuronal (and glial) density in the brain does not accurately represent changes in total number. For example, a decreased density could potentially be accompanied by increases in total volume, equating to no changes in the total number of cells. For this reason, stereological analysis of neuron number, in which density is multiplied by volume, is preferred.

Studies that have attempted to determine whether glial cells are affected by BPA have mainly concentrated on changes to GFAP expression in astrocytes. Early developmental exposure to BPA increased density of GFAP staining in the cingulum at PND 20 (Kunz et al., 2011) and an increase in astrocyte process density in the hippocampus was demonstrated in male rodents exposed during adulthood (Leranth et al., 2008). An increase in GFAP expression has also been demonstrated *in vitro* after application of BPA (Yamaguchi et al., 2006; Miyatake et al., 2006). One study has investigated the effects of BPA on precursor cells of oligodendrocytes, which are responsible for myelination of axons in the central nervous system. *In vitro* treatment of BPA blocked differentiation of oligodendrocyte precursor cells induced by application of

thyroid hormone (Seiwa et al., 2004). Currently, it is unknown whether developmental exposure of BPA *in vivo* has long-lasting impacts on the number of astrocytes or numbers of other types of glia, such as microglia or oligodendrocytes.

In addition to neuroanatomical changes associated with neurons and glia, there is evidence to support the idea that BPA exposure contributes to alterations in synapses and spines. BPA was found to dose-dependently alter synaptic density in the hypothalamus in an *in vitro* model, with an increase demonstrated at a lower dose and decrease demonstrated in a higher dose (Yokosuka et al., 2008). Across several studies, it has also been shown that BPA exposure in adulthood prevents the synaptogenic response to both estrogen and testosterone (Leranth et al., 2008a; 2008b, MacLusky et al., 2005). To elaborate, 50µg/kg/day of BPA administered during adulthood to female nonhuman primates attenuated an estrogen-mediated increase in the number of spine synapses in both the hippocampus and prefrontal cortex (Leranth et al., 2008a). Additionally, the testosterone-induced increases in spine synapses in the prefrontal cortex and hippocampus was not seen if male rats were exposed to 300µg/kg/day of BPA (Leranth et al., 2008b), and female adult rats given the same dose of BPA were found to have an attenuation of estrogen-mediated increases in hippocampal spine density (MacLusky et al., 2005). These findings point to a susceptibility of adult spines to be impacted by BPA, although the ability for exposure during early development to exert similar effects in a long-lasting manner remains unknown.

BPA may perturb different developmental processes, such as proliferation, differentiation, and apoptosis, which may contribute to the previously mentioned

changes in adult neuroanatomy. Several studies support the idea that BPA alters both proliferation and differentiation of neural progenitor cells (NPCs), which can develop into neurons, astrocytes, or oligodendrocytes (Okada et al., 2008; Okada et al., 2010; Kim et al., 2007). In NPCs isolated from the cerebellum, BPA dose-dependently suppressed proliferation and increased cytotoxicity, and these effects were potentially mediated by activation of certain protein kinases and increases in reactive oxygen species (Kim et al., 2007). Further support for the idea that BPA induces apoptosis comes from evidence in which BPA increased reactive oxygen species, calcium, and phosphorylation of mitogen-activated protein kinases, along with activation of caspases, in the hippocampus (Lee et al., 2008). Another potential mechanism for changes in neurons or glia could be due to a BPA-induced rise in glutamate and NMDA receptors in the hippocampus (Kunz et al., 2011; Lee et al., 2008) given that BPA can exacerbate glutamate-induced neuronal death in organotypic slices obtained from PND 8 rats in the CA3 region of the hippocampus (Sato et al,. 2002).

Migration is another developmental process that may be susceptible to perturbations induced by BPA. Subcutaneous injections of 20µg/kg/day of BPA to pregnant mice during early gestation decreased BrdU (which labels dividing cells) in the ventricular zone and increased labeling in the cortical plate when brains from embryos were analyzed at day 16.5, 2 days after the BrdU injection (Nakamura et al., 2006). During this period of development, most neurons radially migrate from the ventricular zone to the cortical plate (which develops into the cerebral cortex). In addition, this same study failed to find any changes in proliferation of precursor cells, leading to the conclusion that BPA exposure resulted in accelerated neuronal migration (Nakamura et

al., 2006). In a later study by the same research group, evidence for accelerated migration of somatosensory cortex neurons after BPA exposure was evident at 3 weeks after birth, but these differences disappeared by 12 weeks after birth (Nakamura et al., 2007). BPA exposure also resulted in abnormal thalamocortical and corticothalamic projections in the third postnatal week that persisted until the 12th postnatal week (Nakamura et al., 2007). This study suggests that while compensation prior to adulthood can occur with respect to migration of neurons in the cortex, BPA-induced changes in projections of axons are long-lasting. Together, the available evidence discussed in the preceding paragraphs supports the potential of BPA to alter the trajectory of developmental processes that are necessary for normal numbers of neurons and glia in adulthood.

As discussed above, some studies have examined whether developmental BPA alters neuron number in adulthood in several brain areas and other studies have attempted to discern whether developmental processes that contribute to this endpoint are disturbed. To date, no studies have investigated whether developmental exposure to BPA alters adult neuron and glia number in the medial prefrontal cortex, a brain area important for cognitive functioning. By the 10th day after birth (which corresponds to the end of dosing in the current study), neurons in the rat prefrontal cortex have undergone a myriad of developmental processes such as proliferation, migration, differentiation, and apoptosis so that most of the layers of the prefrontal cortex are anatomically distinguishable (van Eden & Uylings, 1985). Although anatomical development is by no means complete, particularly with respect to differentiation, apoptosis, and dendritic alterations (van Eden & Uylings, 1985; Nunez et al., 2001; Koss et al., 2010), the rate

and variety of processes in early development suggest that this is a critical period that could be particularly sensitive to toxicological substances or other insults.

Previous evidence from our lab suggests that there are sex differences in the development of the cortex. For instance, the trajectory of apoptosis in the developing posterior cortex (which later develops into the primary visual cortex) is different between males and females, in that males exhibit a larger peak at PND 7 and females exhibit a larger peak at PND 11 and 25 (Nuñez et al., 2001). While no studies have measured apoptosis during early development in the prefrontal cortex, this previous work points to a possibility that sex differences in timing of apoptosis in early development could occur, potentially affecting adult neuron and glia number.

Other work from our laboratory has shown that anatomy of the medial prefrontal cortex (mPFC) develops in a sex-specific manner past early development. For example, between PND 20 and 35, both sexes showed dendritic growth, while dendritic pruning between PND 35 and 90 was only apparent in females (Koss et al., 2010). Furthermore, while neuron number in the mPFC is similar between male and female rats at PND 35, there are sex differences in neuron and glia number in adulthood (Markham et al., 2007). Emergence of sex differences in adulthood is characterized by neuron loss that occurs between PND 35 and 90, which is present in both sexes, but more exaggerated in females. Consequently, females have fewer neurons and glia in the mPFC than males in adulthood (Markham et al., 2007). It is likely that endogenous hormones, such as estrogen, progesterone, and testosterone are contributing to these adult sex differences, and work in our lab is currently underway to directly test this hypothesis by removing gonads prior to puberty and assessing whether adult sex

differences in prefrontal cortex anatomy are altered. Although the preceding discussion is focused on changes that occur after administration of BPA in the current study, endogenous hormones during early development may provide a foundation for these later changes. Hence, exposure to BPA during early development may produce alterations in the timing, intensity, or direction of sex-dependent developmental changes that occur between adolescence and adulthood. Accordingly, these differences may contribute to a sex-dependent response to endocrine disruptors, such as BPA. The current study aims to clarify whether BPA alters adult neuron and glia number in the mPFC in a sex-dependent manner.

Methods

Histology

After the last day of training in the previously described radial arm maze task in Chapter 3, food was returned ad libitum immediately. 2-5 weeks after the completion of training in the behavior task, rats were sacrificed. On the day of sacrifice, rats were injected with 100mg/kg sodium pentobarbital and perfused with 0.1 M phosphate buffered saline (PBS) for 4 minutes followed by 4% parafomaldehyde in PBS for 5 minutes. Brains were removed and weighed. All brains were stored in the 4% paraformaldehyde solution for nine days, transferred to a 30% sucrose solution for 3 days, and then sectioned on a freezing microtome. Every fourth 60µm section was mounted on slides and then stained with Methylene Blue/Azure II on the following day.

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 (Markham et al., 2007; Van Eden & Uylings, 1985). The boundaries of PL/IL within a section were determined through cytoarchitectural characteristics of these areas and the cortical regions bordering them. Within and between cohorts, boundaries were randomly redrawn to confirm consistency within 5% of the original parcellations. Prefrontal gray matter volumes were parcellated from the most anterior section where the underlying white matter appears 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 the layers 2/3 and layers 5/6 separately. Additionally, white matter adjacent to the mPFC was parcellated. All drawings were scanned into a computer, areas were measured with Image J, and post-shrinkage thickness for each layer was measured 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.

Neuron and Glia Number

Total numbers of neurons and glia were determined as described previously (Markham et al., 2007; Koss et al., submitted) using the optical disector with the Stereoinvestigator program (Microbrightfield). Neuron and glia density were quantified separately for the layers 2/3 and layers 5/6 of the mPFC. The computer program randomly chose counting frames, which measured 35µm x 35µm (width x height), within parcellated boundaries. Guard zones were set at 1um at the top and bottom of each section. A cell was counted only if the bottom of the cell was within the volume of the

counting frame. Neurons and glia were distinguished based on differences in morphological, size, and color characteristics. At least 400 neurons and 140 glia were counted from the layers 2/3 and the 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 for each animal were multiplied by the volume of the structure for each animal to determine total number of neurons and glia for individual layers.

Statistical Analysis

Analyses of neuroanatomical measures were performed using ANOVAS that included all of the treatments. Cohort was used as a covariate in all of the comparisons due to significant differences between the two cohorts ($p<.05$) in most measures. . Due to the expectation that males and females may exhibit different responses to BPA, females and males were analyzed separately when assessing potential effects of treatment. In situations where effects of treatment were found when analyzing all dose groups, ANOVAS with treatment as an independent factor and cohort as a covariate were utilized to compare individual BPA treatment groups to the control group. ANOVAs with sex as the independent factor and cohort as a covariate were also performed between male and female controls to determine whether sex differences were apparent. Statistical significance was set at $p<0.05$.

Results

Cortical Volume

Results for cortical volume are shown in Figure 5. There were no significant changes in volume of layers 2-3 or layers 5-6 in females. However, in males, there were trends towards increases in volume of layers 2-3 and layers 5-6 when all

treatments were included in the analyses (p=.06). In layers 2-3, males exposed to 400µg/kg/day displayed a significant larger volume when compared to males exposed to 40µg/kg/day (p<.05). Additionally, there was a weak trend for increased volume in the 400µg/kg/day compared to the group that received 4 µg/kg/day (p=.09). In layers 5-6 in males, there was a significant increase in volume in males that received 400µg/kg/day compared to the 4µg/kg/day group (p=.05). Also, there were weak trends towards an increase in volume in the 400µg/kg/day group compared to the control group (p=.09) and the 40µg/kg/day group (p=.10). There were no sex differences in volume in layers 2-3 or layers 5-6 in controls. Samples sizes for each treatment and for each sex were as follows: 0µg/kg = 8, 4µg/kg = 10, 40µg/kg = 13, 400µg/kg = 11.

Neuron Number

Results for neuron number are shown in Figure 6. There were no significant treatment effects in females in neuron number in layers 2-3 or layers 5-6 (Figure A and B, respectively). In contrast, there was a significant treatment effect in males in layers 5-6 when all treatments were included in the analysis (p<.05, Figure 6B). Posthoc tests revealed a significant increase in neuron number in males in layers 5-6 that received 400µg/kg compared to controls (p=.05) and the 4ug/kg group (p=.07) Comparison of values obtained in control males vs. males that were exposed to 400µg/kg showed that the percent increase in neuron number was approximately 15%. Although analysis did not reveal a significant effect of treatment in males in layers 2-3 (Figure 6A), the pattern of increased neuron number in the 400µg/kg group was similar to that seen in layers 5-6. No significant sex differences were detected when males and females that received 0ug/kg/day were compared. Samples sizes for both sexes in layers 2-3 were 0µg/kg =

8, 4µg/kg = 9, 40µg/kg = 11, 400µg/kg = 11 and samples sizes for both sexes in layers 5-6 were 0µg/kg = 8, 4µg/kg =10, 40µg/kg = 13, 400µg/kg = 11.

Glia Number

Results for glia number are shown in Figure 7. There were no significant treatment effects in females in glia number in layers 2-3 or layers 5-6. In contrast, there was significant treatment effect in males in layers 5-6 when all treatments were included in the analysis (Figure 7B, $p<.05$). Posthoc tests revealed a significant increase in glia number in males in layers 5-6 that received 400µg/kg compared to controls and the 4µg/kg group ($p<.05$). The percent increase of glia number in the 400µg/kg/day group compared to the control group was approximately 19%. Although analysis did not reveal a significant effect of treatment in males in layers 2-3 (Figure 7A), the pattern of increased glia number in the 400µg/kg group was similar to that seen in layers 5-6. Samples sizes for both sexes in layers 2-3 were 0µg/kg = 8, 4µg/kg = 10, 40µg/kg = 13, 400µg/kg = 11 and samples sizes for both sexes in layers 5-6 were 0µg/kg = 8, 4µg/kg =10 , 40µg/kg = 12, 400µg/kg = 11.

White Matter Volume

Results for white matter volume are shown in Figure 8. There were no significant treatments effects in females in white matter. However, there was a weak trend towards a treatment effect in the males ($p=.09$). Posthoc tests revealed that this trend was due to a significantly smaller volume in males that received 4µg/kg compared to those that received 400µg/kg BPA ($p<.01$). No other differences occurred between other treatment groups in males. Comparison between control males and females

revealed a sex difference ($p<.05$). Samples sizes for each treatment and for each sex were as follows: 0µg/kg = 8, 4µg/kg = 10, 40µg/kg = 13, 400µg/kg = 10.

Discussion

The results found in this study provide the first evidence that early exposure to bisphenol A can induce increases in neuron and glia number in the prefrontal cortex of males. This effect is only seen at the highest dose used in the current study, 400µg/kg. Importantly, this dose is particularly relevant to exposure that occurs in humans given the evidence that 400µg/kg produces serum blood levels in female adult rodents and female rhesus monkeys that are similar to those observed in female humans (Taylor et al., 2011). Additionally, effects on neuron number in males in the 400µg/kg dose were only significant in layers 5-6, although the direction of changes in layers 2-3 were similar to that seen in layers 5-6. The importance of increased neuron number in males become apparent when considering that this neuroanatomical measure is organized early in life and is less plastic than other changes. Unlike other neuroanatomical alterations, such as those in dendritic spines or glia number, there are no known pharmacological or environmental interventions (i.e. environmental enrichment) that could be given either during this period of development or later in life to normalize neuron number. Normal proliferation and apoptosis in neurons occurs during specific periods of development in contrast to glia, in which proliferation occurs throughout life. Additionally, alterations in neuron number is unlike potential changes in neurotransmitter systems, where pharmacological medications could be used to attenuate long-lasting effects.

The increase in neuron number in males reported here is in contrast with predictions made by comparisons with previous studies. Most of the studies that examine changes that may be related adult neuron number in the current study may suggest that neuron number would be decreased. However, there are important differences, such as dose of BPA and brain areas examined, between the current study and the past studies that could account for differential effects. For example, a study by Kunz et al., (2011) found decreased neuronal density in the hippocampus after exposure to BPA. The estimated dose used in Kunz et al. (2011) was 70µg/kg/day. The dose most similar to this in the current study was 40µg/kg/day, where no differences in neuron number were observed. Also, this study only analyzed neuronal density and did not account for changes in volume. In the current study, trends in increases in volume suggest that volume changes in the prefrontal cortex may at least partially contribute to the changes in neuron number. This idea of changes in volume accounting for changes in neuron number is also supported by a study in which alterations to neuron number in the locus coeruleus in response to BPA exposure corresponded to changes in volume (Kubo et al., 2001). Therefore, it is important for studies that evaluate BPA-induced alterations in neuronal density or neuron number must consider that alterations in volume may also occur.

The mechanism responsible for the increase in neuron and glia number in males in layers 5-6 is unknown. Alterations to apoptotic, proliferation, or differentiation processes could all lead to changes in cell number. As mentioned in the introduction, only one study has attempted to discern whether alterations to proliferation would occur after exposure to BPA and they failed to find any significant effects on this measure

(Nakamura et al., 2006). However, this study only examined one dose of BPA (20µg/kg/day) which most closely resembles the 40µg/kg/day dose that did not result in any changes in neuron or glia number in the prefrontal cortex. Therefore, more studies are needed to examine whether expose to 400µg/kg/day alters proliferative process in males.

Cell death is another process that may be contributing to the differences seen in the present study. Several i*n vitro* studies have reported increases in measures of cell death in the hippocampus (Lee et al., 2008; Sato et al., 2002), which is contrary to what would be predicted in the current study. However, there is lack of data on what concentrations of BPA used in neuronal cultures would equal those used in *in vivo* studies. One potential reason why males may be susceptible to alterations to cell number by exposure to BPA is that males exhibit a peak in apoptosis in the early postnatal period (PND 7) in the posterior cortex, while females have peaks in apoptosis later in development (Nuñez et al., 2001). Importantly, this peak in apoptosis in males corresponds to the time in which BPA was administered in the current study. While it is not known whether similar time course also exists in the prefrontal cortex, it is possible that BPA exposure during the early postnatal period may attenuate an early apoptotic peak in males.

The full implications of an increase in neuron number in the prefrontal cortex in response to early BPA exposure are currently unknown. However, the direction of changes in neuron number found in the current study is similar to what has been seen in a preliminary study of autism. Courchesne et al (2011) found that human male children with autism had a significantly larger amount of neurons in the prefrontal cortex

(Courchesne et al., 2011). In this study, the percent increase in neuron number in the dorsolateral prefrontal cortex in autistic male children was 79%, which is considerably larger than the increase observed in the current study (15%). Given the large discrepancy in these values between the current study and Courchesne et al (2011), it is unlikely that BPA exposure alone leads to development of autism. The biological basis of autism likely arises from a variety of neuroanatomical and functional changes in the brain. However, BPA exposure in males during early development may be detrimental to populations that already present with other suggested risk factors for autism, such as advanced parental age, maternal exposure to known toxicants, and maternal viral infections, and genetic predispositions (Grabrucker, 2012; Devlin and Scherer, 2012). It is widely know that human males are much more likely to be diagnosed with autism than females (Werling and Geschwind, 2013). This bias of autism in males is consistent with the current study in which only males exhibited an increase in neuron number in response to BPA exposure. More studies are needed to determine whether BPA exposure leads to neural changes that are seen in developmental disorders, such as autism.

The increases in glia number in the prefrontal cortex of males exposed to 400ug/kg/day could be due to changes in astrocytes, oligodendrocytes, and microglia due to the fact that the Nissl staining 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 on GFAP density are unknown. No studies to date have investigated whether BPA directly impacts oligodendrocytes, but one *in vitro* study

did report dose-dependent alterations in neural progenitor cells (NPCs), which can develop into astrocytes, oligodendrocytes, and neurons (Okada et al., 2008). In order to confirm that this change was associated with changes in glia, this study did demonstrate BPA-induced increases in NG2-positive oligodendrocyte precursor cells. However, this study reported some decreases in NPCs at higher concentrations of BPA (Okada et al., 2008), but as stated previously, it is difficult to ascertain what concentrations of BPA in culture studies would correspond to doses of BPA used in *in vivo* studies or exposure that occurs in humans. Therefore, more studies using *in vivo* administration of BPA are needed to determine what types of glia are contributing to the increases seen in this current study.

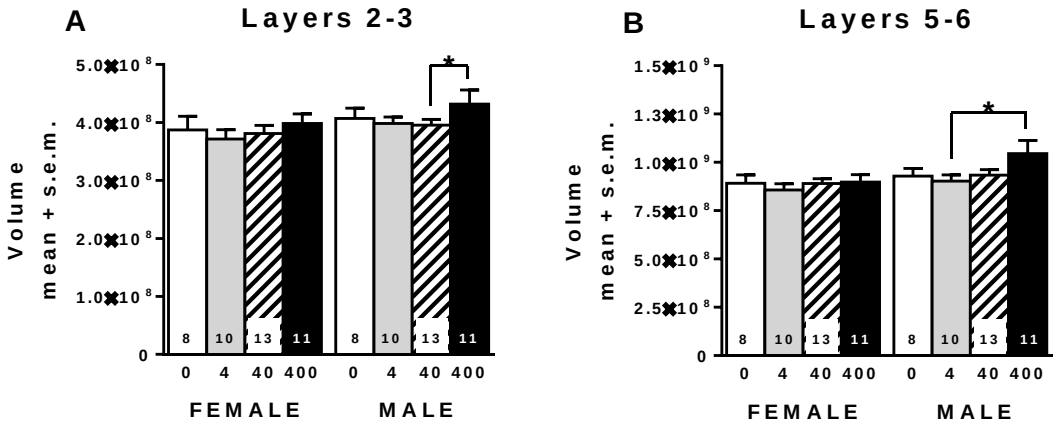
No significant sex differences were seen in either neuron or glia number in the mPFC when male and female controls were compared, even though our lab has previously reported sex differences or indications for sex differences when assessing these measures in adulthood (Markham et al., 2007; Koss et al., 2012). Similar to the previous chapter where no sex differences were found in radial arm maze behavior, this discrepancy may be due to handling of both the dam during gestation and pups during early postnatal development that was necessary for the purposes of dosing in the current study. The evidence for different responses between the sexes after early postnatal handling is seen in release of ACTH after exposure to shock (Erskine et al., 1975), area of the corpus callosum (Berrebi et al., 1988), and turnover of serotonin in prefrontal cortex (Duchesne et al., 2009). Also, it unknown how administration of oil during postnatal development impacts sex differences in gray matter volume, neuron number, and glia number in the mPFC.

Lastly, in addition to alterations seen in glia and neuron number in the prefrontal cortex of male rats, a significant increase in white matter volume was seen in males in the 400µg/kg compared to the 4µg/kg/day treatment group. No significant differences were seen in any treatment group compared to the control group, indicating that the difference was due to a non-significant decrease in the 4µg/kg and a non-significant increase in the 400µg/kg group. To date, only one study has assessed potential changes in myelination in the cortex or corpus callosum (Kunz et al., 2011). This study failed to find any changes in staining for myelin basic protein with exposure to BPA. However, the only dose of 70µg/kg/day used in this study most closely resembled the 40µg/kg/day group in the current study, which had a similar white volume to the control group. The difference in white matter volume between the two BPA-treated groups could be due to alterations in the number of oligodendrocytes given that a significant increase in glia number was apparent in males treated with 400µg/kg compared to the 4µg/kg group.

In the mPFC, exposure to 400µg/kg/day increases neuron number and glia number in males only. The reason for this particular susceptibility in males is unknown, given the evidence that BPA can bind to a variety of receptors, including estrogen, androgen, and thyroid receptors (see Welshons et al., 2006). Future studies are needed to assess the mechanism for these changes and the specific type of cells (as in the case of glia) that are contributing to these changes.

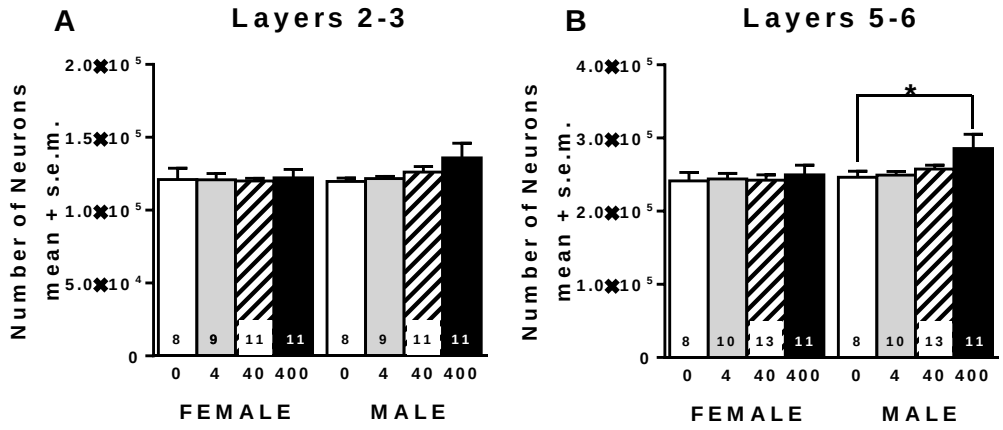
Figures

Figure 5. Volume of the Prefrontal Cortex



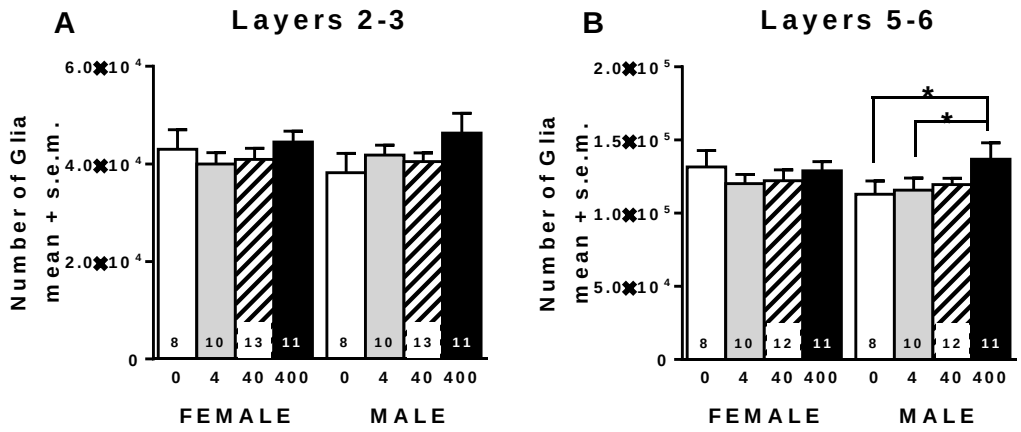
BPA did not alter cortical volume in females. There were trends towards treatment effects in males in both layers 2-3 and layers 5-6 (p=.06). *p<.05

Figure 6. Neuron Number in the Prefrontal Cortex



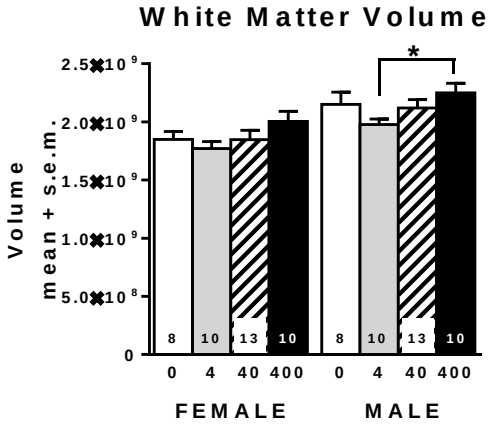
BPA did not alter neuron number in females. There was a significant increase in neurons in males when comparing the control vs. the 400µg/kg. *p<.05

Figure 7. Glia Number in the Prefrontal Cortex



BPA did not alter glia number in females. There was a significant increase in neurons in males when comparing the control to the 400µg/kg or the 4µg/kg group. *p<.05

Figure 8. White Matter Volume



BPA did not alter white matter volume in females. There was a significant increase in white matter volume in males when comparing the 4µg/kg males to the 400µg/kg males. *p<.05

Chapter 5:

General Conclusions

The current studies demonstrated that BPA has no effects on measures commonly used in toxicology to assess general growth and development and minimal effects on cognitive behavior, as assessed in the radial arm maze. However, significant increases in both neuron and glia number in the medial prefrontal cortex in males only in layers 5-6 suggest that this chemical has long-lasting effects on the anatomy of the prefrontal cortex. While the current safe reference dose for humans used by the U.S. EPA and FDA is 50µg/kg/day, measures of blood serum levels in rodents and non-human primates indicate that exposure to larger doses of BPA (400µg/kg/day) correspond to blood levels seen in humans (Taylor et al., 2008). This suggests that the dose of 400ug/kg/day reflects exposure that may occur in humans.

The use of different doses in the current study was integral in evaluating the potential of BPA to disrupt T4 levels, behavior, and anatomical changes in the prefrontal cortex. Many previous studies that have examined similar endpoints in adulthood only utilize one or two doses of BPA, potentially missing the ability to detect specific changes that occur only at certain doses. In particular, the results of T4 levels at weaning showed that BPA has the potential to impact physiology in a nonlinear manner, an idea that has been highlighted in recent reviews describing the effects of endocrine disruptors on numerous endpoints (Vandenberg et al., 2009; Vandenberg et al., 2012; Vom Saal and Hughes, 2005). While this theory is not new to the field of endocrinology, it has not been fully integrated into the field of toxicology. The current practices by

agencies that are responsible for determining safe reference doses for toxicological chemicals review evidence to determine the lowest observed effect level and assume that doses below these levels are safe. Therefore, it is important for federal agencies to adopt new practices when determining safe reference doses for human exposure to chemicals.

In comparing the results from the different chapters, males seemed particularly susceptible to perturbations induced by BPA on behavior and on the anatomy of prefrontal cortex in the current study. The mechanism that contributes to the male susceptibility is unknown, as there is evidence that BPA binds to receptors of many hormones, such as thyroid, estrogen, and androgen receptors (Welshons et al., 2006; Wolstenholme et al., 2011). In contrast to the male susceptibility seen with the anatomy and behavior, significant differences were seen in females with regards to changes in T4, while males showed similar patterns of changes in T4. Therefore, although no differences in behavior or anatomy were seen in females, it is possible that BPA could also impact functioning of other neural measures in females. However, more work needs to be done in order to determine that BPA directly impacts T4 serum levels during the time at which dosing occurs.

Although some indications for alterations in radial arm maze behavior were seen in males, it is unlikely that the increases in neurons and glia were directly responsible for these behavioral changes. Significant behavioral changes were seen in the 40ug/kg/day group, but neuron and glia number alterations were seen in the 400ug/kg/day group, suggesting that the minor behavioral effects of BPA on radial arm maze performance in males may be due to other changes in brain anatomy or

functioning in the prefrontal cortex or the hippocampus. The hippocampus is another structure which may mediate the minor behavior effects seen in males in radial arm maze behavior, given the overwhelming evidence that this brain area is integral for learning of spatial cognitive tasks.

Although only slight modifications in cognitive behavior were apparent with early exposure to BPA, there is evidence that BPA alters anxiety-related and social behaviors. For example, many studies that have investigated the impact of BPA on these types of behaviors as assessed in open field and elevated plus maze (Patisaul & Bateman, 2008; Cox et al., 2010; Ryan & Vandenbergh, 2006; Adriani et al., 2003; Gioiosa et al., 2007; Rubin et al., 2006; Fujimoto et al., 2006; Kubo et al., 2001; 2003). These types of behavior are relevant to idea that BPA may induce changes that may increase the susceptibility for developmental neurological disorders, such as autism, schizophrenia, and ADHD. In fact, several reviews have highlighted the potential for endocrine disruptors, such as BPA, to contribute these disorders (de Cock et al., 2012; Brown, 2009). In conclusion, future studies are needed to determine whether the effects of BPA on different types of behavior and whether the neuron and glia changes demonstrated in the current study lead to behavioral changes that correspond to those seen in neurological disorders.

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