NEURAL EFFECTS OF EXPOSURE TO THE ENVIRONMENTAL CHEMICAL, BISPHENOL A, DURING DEVELOPMENT

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

NEURAL EFFECTS OF EXPOSURE TO THE ENVIRONMENTAL CHEMICAL, BISPHENOL A, DURING DEVELOPMENT

by

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Adviser: Victoria Luine, Ph.D.

Exposure to Bisphenol A (BPA), an environmental chemical, has been linked to changes in physiology, neural development, and behavior. The focus of this study was to determine the effects of BPA exposure, during a short developmental window, on physiology, activity, anxiety, cognition, and neurochemistry. In prenatal study, dams were administered 100 μg/kg/day orally, from gestational day 16 to parturition. Postnatal study pups received subcutaneous injection of 60 or 100 μg/kg BPA from postnatal day 0 to 6. All pups were weighed, examined for evidence of vaginal opening, and, at adulthood, performed behavioral tasks measuring locomotor activity, anxiety, and visual and spatial memory. Brain monoamines were measured using high performance liquid chromatography in the postnatal group. Prenatal BPA contributed to low juvenile body weight in both sexes and adult overweight in male subjects. Hyperactivity and memory deficits were observed in both sexes of BPA treated subjects. Postnatal 100 μg/kg BPA females experienced delayed vaginal opening, less anxiety behavior in elevated plus maze, and spatial memory impairments. BPA treated subjects of both sexes had increased norepinephrine and dopamine turnover in basolateral amygdala and hippocampus, areas which are implicated in

anxiety and cognition, respectively. The data suggests that BPA exposure during perinatal life causes disruptions in physiology, behavior, memory and neurochemistry that persist to adulthood. In addition, postnatal effects of BPA may be mediated by alterations in central monoaminergic function.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Prenatal

Postnatal

I. Introduction

Bisphenol A (BPA) is a constituent of polycarbonate plastics, coatings and resins, and is found in many common products, including the linings of food cans, resin dental fillings and plastic food containers. BPA acts as a fungicide. According to the BPA Safety Committee (Japan), approximately 2 million tons of BPA are produced yearly, worldwide. Over time, the compound leaches from plastic into food materials and ground water. BPA has been shown to interfere with thyroid function (Zoeller, et al, 2005), has carcinogenic properties (Keri, et al, 2007), and contributes to many ill health effects in humans (Lang, et al 2008). When ingested, BPA also produces mildly estrogenic effects through its interaction with the estrogen receptor (Choi and Jeung, 2003).

Bisphenol A- Exposure and Chemical Properties

BPA has wide use and is found in low concentrations in ground water and food products. BPA is a very weak agonist to the classical estrogen receptor α, with an affinity of approximately 1:2000 (Krishnan, et al, 1993). Bisphenol A has also been found to have anti-thyroid (Sun, et al, 2009) and anti-androgenic effects (Sohoni P & Sumpter JP, 1998). Although the safety of BPA has been widely studied by US government agencies that have determined it to be safe (no adverse effects) at exposure of up to 50 mg/kg/day per day, the safe exposure level in the European Union has been calculated as 0.05 mg/kg per day, and BPA-containing products have been banned in Canada. There is argument that levels of consumption needed to show adverse effects are much higher than average exposure. The average groundwater concentrations of BPA range from 0.06 to 1.9 μg per liter, however, in the developed world, we have much higher (unmeasured)

exposure through our processed food containers (plastic and metal) and dental materials. Studies of food containers have found measurable levels of BPA in high-fat products (Cao, et al, 2008) and acidic foods like colas and canned tomatoes (Cao, et al, 2009; Grumetto, et al, 2008). BPA has been detected in infant formula in both canned form, 0.1 to 13.3 ng/ml (Biles, et al, 1997) and powder form, 45-113 ng/ml (Kuo & Ding, 2004). Polycarbonate baby bottles leached 8.4 ng/ml of BPA following simulated food contact and hot water washing (Brede, et al, 2003). Bisphenol-containing compounds, tetrabromobisphenol A and tetrachlorobisphenol A, are used as flame retardant in clothing, linens and home goods such as carpeting. Studies of fluids and tissues of adult humans have found urinary levels of BPA as high as 2.82 ng/ml (Kim et al, 2003), breast milk, 1.9-7.3 ng/ml (Ye, el al, 2006), amniotic fluid, 8.3 ng/ml (Ikezuki, et al, 2002) and placental tissue, 11.2 ng/ml (Schonfelder, et al, 2002).

Levels in humans younger than the age of 6 have not been measured. Estimated adult exposure is 0.6 to 71.4 micrograms/day (Ouchi & Watanabe, 2002), and is likely to be higher in infants due to a greater feeding-to-weight ratio than that of an adult. The exposure of fetuses and infants to bisphenol A is also believed to be higher and more prolonged than that of adults, because of the multiple sources of exposure- direct: placenta; dietary: breast milk, infant formula, water, polycarbonate bottles; and environmental: plastic toys, carpeting, flame-retardant clothing and bedding. The estimated exposure of infants from polycarbonate bottles alone is believed to be 13 micrograms/kg/day (European Food Safety Authority Opinion, 2007). The glucuronide conjugating system, responsible for the breakdown and elimination of BPA and other drugs from the body, has not fully developed in infants and young children (Allegaert, et al, 2009), which

prolongs the time required for BPA clearance. Studies have suggested that the plasma concentration of BPA in infants will be 11-fold than that of an adult administered the same dose (Edginton A & Ritter L, 2009).

Hormones in Brain Development

Prolonged exposure to estrogenic compounds, especially during development, is of great concern. During development, estrogen is responsible for sexual differentiation of the brain (Levine,1971). Estrogen is especially important in creating sexual dimorphism of the hypothalamus, preoptic area, which are responsible for regulating the hypothalamic-pituitary-gonadal axis, and hippocampus, an area of the brain important to learning and memory (Fitch and Denenberg, 1998). In the rat, the critical period for sexual differentiation begins 18 days after conception, and continues until postnatal day 7 (MacLusky and Naftolin, 1981). Of the two estrogen receptor sub-types, ERα is expressed to a higher degree early in life, and ERβ expression increases later in life (Albertazzi & Purdie, 2001).

Estrogen is essential during development for the masculinization and defeminization of the brain. In mammals, the default sex is female, and a fetus will only differentiate into a male under the influence of gonadal hormones. Masculinization is an enhancement of male characteristics, while defeminization is a suppression of female characteristics. In rats, during a critical period in development, beginning 4 to days before birth and continuing to approximately postnatal day 7, the brain is particularly sensitive to gonadal hormones (MacLusky and Naftolin, 1981). In males, testosterone, produced by the testes, travels through the bloodstream to the developing brain. The

testosterone is then converted into estrogen through the actions of the enzyme aromatase. In females, estrogen is sequestered by α- fetoprotein, a protein produced in the liver, protecting the female brain from estrogen's masculinizing effects (Bakker, et al, 2006).

Estrogen activates receptors on neurons and initiates a number of genomic events. For example, estrogen inhibits apoptosis in sexually dimorphic brain regions, such as SDN-POA (sexually dimorphic nucleus of the preoptic area), an important region for male sexual behaviors (Dohler, et al. 1984). In the event that a female brain is exposed to testosterone (aromatized in the brain to estrogen) during this critical period, the brain will assume some masculine features, such as an enlarged SND-POA. Similarly, if a male is deprived of testosterone through castration during the critical period, the brain will have a more feminine morphology (Jacobson, et al, 1981). Females with masculinized SDN-POAs have delays and difficulties in lordosis behavior (Dohler, 1991). Males with feminized SDN-POAs have an absence or delay in mounting of females, and may exhibit lordosis behavior (Rhees, et al, 1999; Ogawa, et al, 1997). The activation of estrogen receptors also contributes to sex-specific cell loss in the AVPV (anteroventral periventricular nucleus). This nucleus, responsible for the ovulation-triggering LH surge in females, is significantly smaller in males (Sumida, et al, 1993). Exposure to androgen during development exerts an organizational effect on the brain, leading to absence of LH surge, anovulation, and sterility (Barraclough C, 1961; Barraclough and Gorski, 1961).

Differences in the hippocampus of males and females also exist. Males tend to have a greater number of mossy fiber synapses and more spines on the apical dendrites in the CA3 pyramidal

cells of the hippocampus (Madeira, et al, 1991). This difference may underlie the observation that males perform better than females on spatial memory tasks (Barrett and Ray, 1970; Luine, 2008)---tasks in which the hippocampus plays an important role. Disturbing the hormones that influence this difference may cause a change in behavioral patterns. Removal of testosterone via gonadectomy abolishes sex difference on 12-arm radial maze, a spatial memory task (Gibbs and Johnson, 2008).

Sex differences are also seen in anxiety behavior. Females, in elevated plus maze performance, are significantly less anxious than males (Johnston and File, 1991). Neonatally castrated males, however, have performance in the maze comparable to females (Lucion, et al, 1996). In prenatal stress studies, females' anxiety increased to a level comparable to males (Bowman, et al, 2004). Neurochemical data was altered toward the male profile in these prenatally stressed females. Contextual fear extinction, more pronounced in female rats than in male rats, is enhanced by administration of an estrogen receptor beta agonist (Chang, et al, 2009).

Thyroid hormone during development is crucial in the migration and differentiation of neurons (Horn & Heuer, 2009). Neonatal hypothyroidism has also been associated with hypomyelination (Bernal, 2002), memory deficits and low IQ (Zoeller & Rovet, 2004). Prenatal neurological hypothyroidism contributes to severe mental retardation, deaf-mutism and cerebral palsy (Porterfield & Hendrich, 1993). Exposure to propylthiouracil (PTU), a chemical which inhibits thyroid hormone synthesis, blocks brain angiogenesis (Zhang, et al, 2009).

Hormonal Disruption Induced by Environmental Chemical Exposure

Throughout life, as in during development, hormonal release and action is tightly regulated by the brain and gonads. During the neonatal period, surges and/or elevations of hormone levels act to "organize" the brain; either by providing neuroprotection or inducing apoptosis in targeted regions of the brain (McEwen, et al, 1977). These sexually dimorphic brain changes are important in laying the groundwork for behavior, as well as processes crucial to reproduction (eg. LH surge). In a study conducted by Tanaka, et al (2006), it was found that BPA exposure may inhibit hormonal surges. Two hours after birth, male rat pups experience a testosterone surge. Rat dams were exposed to BPA for the entire gestational period. Male pups were sacrificed two hours after birth and serum testosterone was measured. It was found that BPA exposure inhibits the T surge in the neonatal period ---decreased T levels inversely proportional to BPA levels.

In a study by Watanabe, et al (2003), it was found that developmental exposure to BPA may contribute to lasting disruptions in hormonal homeostasis. Pups of dams administered 4, 40, or 400 mg/kg body weight BPA through gavage from GD6 to lactation day 20 had significantly higher plasma testosterone levels at nine weeks old when compared to their control counterparts.

BPA has a much lower binding affinity for the thyroid receptor, but is believed to be a weak T3 antagonist (Moriyami, et al, 2002). Developmental exposure to BPA antagonizes T3 receptor activation and gene regulation, inhibiting postembryonic development in tadpoles (Heimeier, et

al, 2009). In rats exposed to BPA perinatally, however, an increase in T4 and RC3/neurogranin expression, a gene responsive to thyroid hormone (Zoeller, Bansal & Parris, 2005).

Physiological Effects of Bisphenol A Exposure

BPA has the ability to exert organizational effects on both the brain and reproductive organs. These effects may bring about physiological changes, specifically disruption of sexual maturation and homeostatic processes such as body weight. Female rats administered 400 or 600 mg/kg/day of BPA from PND 22 to 42 had significantly decreased body weight, and a slightly delayed date of vaginal opening (400 mg/kg group, not statistically significant). The dosages used in this study were toxic levels (George, et al, 2003). In a study by Rubin, et al (2001), lower doses of BPA (.1 mg/kg and 1.2 mg/kg) administered to dams from GD6 to weaning induced increased body weight at birth, which persisted through adulthood. The high dose BPA offspring had disruptions in their estrous cycles and low plasma LH levels. Bisphenol A exposure on reproductive organs may interfere with cell differentiation, morphology, and functioning of the organs. BPA exposure has been associated with complex endometrial hyperplasia in humans (Hiroi, et al, 2004). Offspring of rat dams administered 25 and 250 ng BPA/ kg/ day from gestational day 9 to postnatal day 6 (via osmotic pump) had decreased wet weight of the vagina and decreased endometrial lamina propria volume. An increased expression of ERα and progesterone receptors were observed in both the luminal epithelium of the endometrium and subepithelial stroma of these pups (Markey, et al, 2005).

Behavioral and Cognitive Effects of Bisphenol A

Learning/Memory

Rats exhibit gender differences in performance of spatial learning tasks. Specifically, male rats tend to perform better on spatial memory tasks in comparison to females (Lebowitz and Brown, 1999). Estrogen, aromatized from fetal testosterone, exerts effects on the hippocampus during development, as mentioned above. These developmental effects may contribute to the gender differences seen in spatial learning tasks. Recent studies have found BPA exposure may induce changes in performance in spatial memory tasks. A study conducted by Carr, et al (2003), measured performance in the Morris water maze following neonatal oral administration of BPA. At low dosage of BPA, the gender-based difference in maze performance was abolished. Generally, males have better Morris water maze performance--- however, low dose female subjects performed just as well as males. In high dose BPA subjects, however, the female subjects showed a defect in maze acquisition. Although they were able to solve the maze, they spent significantly less time in the escape quadrant than control females.

Hyperactivity

In recent decades, the explosion in the numbers of children with attention deficit hyperactivity disorder, first described by Dr. Heinrich Hoffman in 1845, has motivated researchers to look for environmental triggers that may contribute to this increase. Some studies have pointed to the exposure of children to environmental estrogens as a cause of hyperactivity (vomSaal, et al, 2007). It is hypothesized that children, especially males, may suffer long-term deficiencies when exposed to estrogenic compounds. Apoptosis is an essential process during development. Estrogen inhibits apoptosis in certain brain regions during development, and affects the

formation of synapses in other areas of the brain (Maclusky and Naftolin, 1981; Gorski, et al, 1978). During the developmental period, an excess of neurons and synapses leads to less efficiency in the system, and weaker synaptic connections. In a study by Mizuo, et al (2004), administration of BPA (through BPA enriched meal fed to dams), adversely affected the dopaminergic system in rats. They found that the morphine-induced reward effect was enhanced in the subjects given chronic BPA. Hyperlocomotion was also observed in these subjects. BPA subjects showed significantly more activity during both light and dark phases. In a similar study conducted by Ishido (2004), rat pups were administered BPA directly (intracisternal injection), and dopamineric system activity and spontaneous motor activity were measured. Locomotor activity during light and dark cycles was increased with BPA administration. Although there is no conclusive evidence of a causal relationship between exposure to excess estrogens during development and ADHD, it is interesting to note that such a marked increase in locomotion in rats has been noted in these studies.

Neural Effects of Bisphenol A Exposure

As hormones have powerful effects on neural cells, so do environmental chemicals such as BPA. Data suggests that exposure to BPA may produce estrogenic effects, especially when administered during development (Gursoy, et al, 2001). BPA has been shown in various studies to exert genomic and non-genomic effects in cells (Krishnan, et al, 1993). Non-genomic effects are rapid effects through interaction with receptors on the cell surface. BPA alters dopamine release and rapid Ca^{+2} signaling through activation of cell membrane receptors (Yoneda, et al, 2003; Tanabe, Kimoto and Kawato, 2006). Genomic effects are less rapid, and influence gene

expression. The genomic effects of BPA, especially during development, may contribute to permanent structural and functional changes in the brain. In a study conducted by Katoh, et al (2004), ovine anterior pituitary cells were exposed to 10^{-4} M BPA in culture for 24 hours. They found that BPA suppressed GH secretion, blocked GHRH-induced increases in calcium and cAMP concentrations, and decreased cell number in culture. A decrease in cell number, on a concentration-dependent basis, of ovine pituitary cells, was observed. They concluded that BPA suppresses GH release through interference with cellular transduction processes. During development, growth of Purkinje cells in the cerebellum is stimulated by estrogen and progesterone. Shikimi, et al (2004), investigated the effects of BPA on dendritic growth in cerebellar Purkinje cells. Administration of 500 μg per day of BPA into the cerebrospinal fluid from postnatal day 6 to 9 produced an increase in dendritic outgrowth compared to vehicle.

In a study by Rubin, et al (2006), female pups of mouse dams administered 25 or 250 ng/kg/day from GD 8 to PND 18 had a significant decrease in tyrosine hydroxylase (TH) neurons in the rostral periventricular preoptic area, a region important for estrous cyclicity and estrogen-positive feedback. This area in male mice is significantly smaller than in females. Treatment with BPA completely abolished this sex-specific size difference. Female offspring of dams receiving oral administration of 4 or 40 mg/kg/day of BPA from gestational day 6 to PND 20, 3 weeks after birth showed increased concentration of DOPAC, HVA, 5HT, 5HIAA, and HVA/DA ratio in several brain regions (Honma, et al, 2006). In a study by Zhou, et al (2009), it was observed that 20 microgram/kg/day perinatal BPA exposure resulted in long-term potentiation and depression

deficits in the striatum of rats. It is postulated that an alteration in the DA receptors was induced by BPA exposure.

II. Specific Aims

This study will investigate the effects of developmental exposure to environmental chemical BPA on neurochemistry, physiology, behavior and cognition at adulthood. This research aims to investigate effects of lower dose of BPA, which we believe to be more relevant to environmental levels, and to investigate behavioral, neurochemical and physiological measures in both males and females. The following parameters were investigated in two experiments:

Experiment 1- Effects of Prenatal exposure to Bisphenol A on Physiology, Behavior and Cognition

Aim 1: Investigate Cognitive/ Behavioral Effects of BPA Exposure During Development on Adults

a. Anxiety behaviors via elevated plus maze
b. Performance in spatial and visual memory tasks
c. Locomotor activity and exploratory behavior

Based on evidence from similar studies, we hypothesize that female, but not male subjects will have impairments in visual and spatial memory tasks as a result of prenatal BPA exposure. Although our subjects will be exposed to BPA for shorter durations than subjects in previous studies, we hypothesize that our female subjects will have increased anxiety, and the males will have decreased anxiety in elevated plus maze performance, and increased locomotor activity in open field performance.

Aim 2: Investigate Effects of BPA Exposure on physiology

a. Physiological measures of weight, pubertal onset, genital tract

Females have little estrogen exposure in this developmental period. We therefore hypothesize that BPA will act as a weak estrogen agonist in females, which we hypothesize will induce earlier age at vaginal opening. BPA has been shown to cause overweight in female rodents (George, et al, 2003). Based on evidence in past studies we hypothesize that females will have increase in body weight at maturity. Because prenatal and neonatal males have higher levels of estrogen, aromatized from testosterone, we do not expect to observe significant changes in body weight or genital tract morphology of male subjects.

Experiment 2- Effects of postnatal exposure to Bisphenol A

BPA will be given immediately following birth, and the same physiological and behavioral parameters will be investigated as in experiment 1. Because we are administering BPA slightly later, but within the same developmental window, we hypothesize that the effects on physiology and behavior will be similar to those in the prenatal experiment.

Aim 1: Investigate Cognitive/ Behavioral Effects of BPA Exposure During Development on Adults

a. Anxiety behaviors via elevated plus maze
b. Performance in spatial and visual memory tasks

c. Locomotor activity and exploratory behavior

Aim 2: Investigate Neurochemical Effects of Postnatal BPA Exposure on adults

a. Levels and activity of monoaminergic neurotransmitters and their metabolites (NE and MHPG; DA, HVA and DOPAC; 5HT and 5HIAA) will be measured in 6 brain areas: frontal cortex, CA1 of hippocampus, CA3 of hippocampus, medial preoptic area (MPOA), striatum and basolateral amygdala (BLA). These brain areas were chosen because they are areas important in cognition, locomotion, and anxiety behavior; as well as sexually dimorphic brain regions. We hypothesize that we will observe increased monoamine turnover in areas governing anxiety behaviors (BLA) in males, and a decrease of turnover in females. We expect to see decreased turnover of monoamines in areas involved in memory and cognition (CA1, CA3, Frontal Cortex), and decreased turnover in areas involved in locomotion (striatum). We expect to see a masculinization effect in the sexually-dimorphic MPOA, most likely an abolishment of sex differences in monoamine levels in BPA treated females.

Aim 3: Investigate Effects of BPA Exposure on physiology

a. Physiological measures of weight, pubertal onset, genital tract

III. **Experiment 1: Effects of Prenatal Exposure to Bisphenol A**

The time period from gestational day 18 to postnatal day 7 in rats is analogous to the period of sexual differentiation of the brain in humans, which occurs mid-gestation (MacLusky & Naftolin, 1981). During this period, BPA crosses the placenta, exposing human fetuses directly through the bloodstream. Exposure to BPA may continue throughout neonatal life through breast milk or exposure to BPA-containing plastics. This study will focus on cross-placental exposure to BPA. Dams will be administered 100 μg/kg body weight daily from gestational day 16 to parturition. We will investigate whether exposure to this compound induces behavioral changes in male and female rat pups when they reach adulthood. Behaviors investigated will include anxiety, locomotion, and memory. Physiological measures will also be taken, including body weight, age at vaginal opening, and gonadal weight.

Research Design and Methods

Breeding-

Adult, Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. and housed in the Hunter College Animal Facility. All procedures were approved by the National Institutes of Health Guide for the Care and Use of Animals and the Hunter College institutional animal care and use committee. Rats were allowed to acclimate to environment for 14 days before mating. Mating was conducted at Hunter College Animal facility in order to eliminate maternal stress during shipping as previously reported by the laboratory (Bowman, et al, 2004). Breeder and offspring animals were raised on standard rat chow. Eight, 3 month old females received mating,

and four, 3 month old, males, were used as studs. Females were housed separately and received vaginal smears each morning around 10 AM to determine estrus cycle day. If a female was in proestrus, a male was housed with the female overnight (beginning around 3-4 PM) in order for mating and pregnancy to occur. The male was removed in the morning, cage checked for vaginal plugs, and the female received a vaginal smear to determine presence of sperm. The date of the first positive vaginal smear was recorded as gestation day 1. Females continued to receive smears and mating until successful mating/pregnancy of at least 8 females occurred.

Treatment Groups and BPA Administration-

Beginning gestation day 12, pregnant dams were given untreated small animal puffs (Kaytee Nutra-Puffs Wild Berry Small Animal), once daily to acclimate dams to eating the treats. Dams were weighed each morning before treat administration for accurate dosing. Beginning gestation day 16 to parturition, control group dams received control puffs, BPA treatment group dams received puffs treated with BPA (100 μg/kg body weight). Dose was measured by micropipette. Puffs were saturated with either ethanol (control) or BPA in ethanol, and allowed to evaporate/ dry completely. Starting at day 22 of pregnancy, dams were checked for delivery of pups at 10 AM and at 2, 4 and 6 PM (animal facility personnel checked animals at 9 AM on weekends). A total of 60 pups were sought (15 pups per sex/treatment group). We obtained 8 litters; thus, more pups were born than anticipated and excess were sacrificed on day 1. We experienced a large loss of pups from the BPA litters. Pups were very small and had difficulties thriving. We also observed some disordered maternal behavior in the BPA dams (scattering of pups around cage, lack of licking and nursing). The surviving pups were reduced to 32 (8 pups per sex/treatment

group) by postnatal day 10. Pups were weaned at 25 days, and group housed by sex, in the Hunter College Animal Facility. As they grew, the number of pups per cage gradually decreased until at adulthood, two adults remained in each cage.

Physiological measures-

Body Weight

Rats were weighed at weaning (juvenile body weight), and once again on the day of sacrifice (adult body weight).

Vaginal Opening

Female rats were inspected daily for evidence of vaginal opening beginning on PND 21. Weights were analyzed by two-way ANOVA (sex x treatment), and date of vaginal opening by one-way ANOVA.

Behavior-

At two months of age, behavioral testing began in 32 rats. Elevated plus maze (anxiety), open field behavior (locomotor activity), memory assessment by object recognition (visual) and object placement (spatial), were conducted, in this order. Behavior was run in 1 cohort with 32 rats per cohort. All mazes, field boxes and objects were thoroughly sprayed with Nolvasan/Coverage spray between subjects to remove any scent. Subjects were counterbalanced by sex and treatment.

Elevated Plus Maze-

Subjects were placed into the elevated plus maze, facing away from the examiner, oriented toward an open arm. Rats were allowed to explore the maze for 5 minutes. Number of entries into open and closed arms, as well as time spent in each arm, were recorded. Timing began after rat has placed 3 paws into the arm (Luine, et al, 2006).

Open Field-

Subjects were placed in the center of an enclosed box marked out with 9" squares (3 squares long, 5 squares across). Rats were allowed to explore the field for 6 minutes. We recorded the number of movements across squares, rearing (standing on hind legs with forelimbs at least 3 cm off of floor), defecations, grooming and wall climbs. Recordings were separated for the first three minutes and last three minutes (Bowman R, Ferguson D, Luine V, 2002).

Object Recognition-

Testing was conducted in the open field box described above, reduced in size (3x3). Rats were placed into the center square of the testing field. Testing consisted of two, 3 minute trials: T1 (sample) trial, and T2 (retention) trial, with intertrial delays of 2 and 4 hours. In the sample trial, two identical objects were placed on one end of the field. The amount of time spent exploring the objects was recorded with digital stopwatch. Exploration included looking (at close distance-2 cm), sniffing, and whisking of the object. In T2, one of the objects was replaced with a novel object. Two separate exploration time measurements, one of the novel object, one of the old object, were recorded. The ratio of (time spent with the new object/total time spent exploring old

and new objects) is used to determine if the rats can distinguish the novelty of the object. Animals are habituated to this task with 1, 10, and 60-minute intertrial delays, prior to testing (Luine V, Jacome L, MacLusky N, 2003).

Object Placement-

Testing was conducted in the open field box, reduced in size (3 squares x 3 squares). Rats were placed into the center square of the testing field. Testing consisted of two, 3 minute trials: T1 (sample) trial, and T2 (retention) trial, with an intertrial delay of 2 hours. In the sample trial, two identical objects were placed on one end of the field. The amount of time spent exploring the objects was recorded with digital stopwatch. Exploration included looking (at close distance-2 cm), sniffing, and whisking of the object. In T2, one of the objects was moved to a novel location in the field. Two separate exploration time measurements, one of the novel location, one of the old location, were recorded. The ratio of (time spent exploring object in the new location/total time spent exploring old and new object location) is used to determine if the rats can distinguish the novel location. Animals are habituated to this task with 10, 40, and 60-minute intertrial delays, prior to testing (Luine V, Jacome L, MacLusky N, 2003).

All behavior was analyzed by two-way ANOVA to determine sex differences, treatment effects, and to determine if there was a significant interaction effect (sex x treatment). Post hoc testing was by LSD test.

Following sacrifice, testes, prostate, uteri and ovaries were harvested and weighed. Weights were analyzed by one-way ANOVA.

IV. Results

Physiological measures-

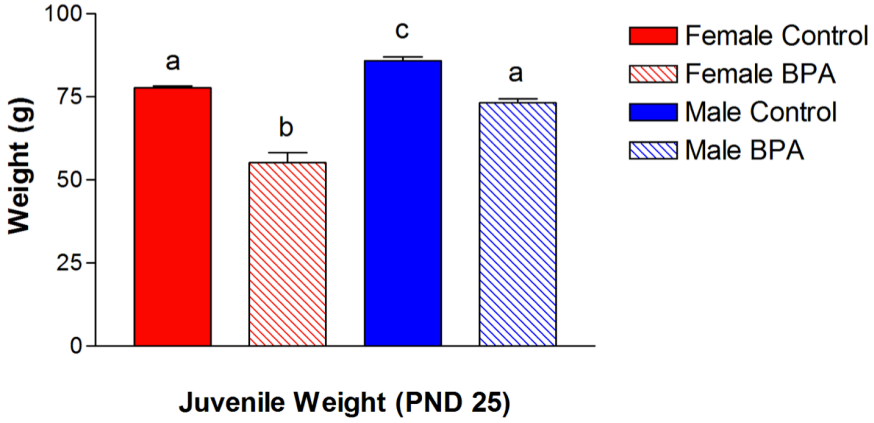
Body Weight (Figure 1 a,b)

Average weight gain during pregnancy in dams receiving BPA treatment is 43g (SEM=0.98). No statistical difference was observed between gestational weight change with respect to control dams, 42.7g (SEM=1.53)---data not shown.

BPA treatment contributed to disturbances in juvenile body weight. Both males and female treated with BPA had significantly lower body weight, 64.2 (SEM= 1.23) when compared to controls, 81.8 (SEM=1.74), $P<0.001$. At postnatal day 40, BPA males were significantly heavier, 480.8 (SEM=6.62) in comparison to control subjects, 446.5 (SEM=9.36), $P<0.001$. No weight differences were observed in female subjects.

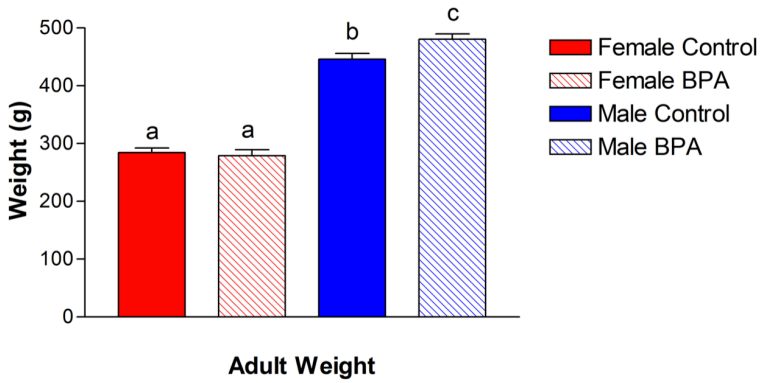
Figure 1

a.



Juvenile body weight. A treatment effect and sex difference were observed in body weight at weaning (postnatal day 25). BPA treated subjects had significantly lower body weight (64.3 m1.23) with respect to control (81.8 m1.25) and males were significantly heavier (79.5 m1.73) than females (66.5 m1.88) . A sex-treatment interaction, $F(3,47)= 7.99$, $P<0.01$, was also observed. Treatment $F(1,49)= 101.10$, $P<0.001$, Sex $F(1,49)=55.93$, $P<0.001$.

b.



Adult body weight. A sex difference were observed in adult body weight, F(1,49)= 377.09 , P<0.001. Males were significantly heavier (463.6m1.73) than females (281.8 m1.88) . A sex-treatment interaction, F(3,47)= 4.50 , P<0.05, was also observed.

Vaginal Opening

No significant differences were observed in date of vaginal opening (data not shown).

Organ Weight (Table 1)

No significant difference in weights of ovaries/uteri, and testes.

Table 1

Reproductive Organ Weights

Organ Weights	Control	BPA
Female	0.840 (0.067)	0.842 (0.001)
Male	3.779 (0.088)	3.93 (0.076)

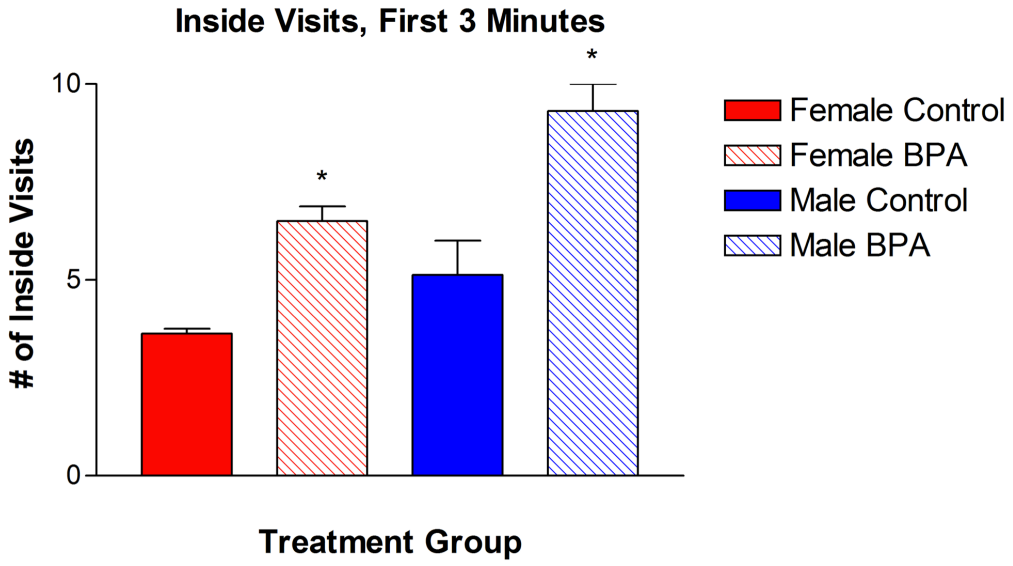
Behavior-

Open Field (Figures 2a,b, 3)

Male and female BPA treated subjects had significant alterations in locomotor behavior. These subjects had significantly increased number of inside square visits, 8 (SEM=1.35) and rearing, 7.375 (SEM=1.38) with respect to control subjects, 5.19 (SEM=0.95) and 4 (SEM=0.979), $P<0.05$. A sex difference was also observed in inside square visits. Males visited inside sectors significantly more, 8.125 (SEM=1.347) than females, 5.25 (SEM=0.952), $P<0.05$, in the last three minutes of the open field task.

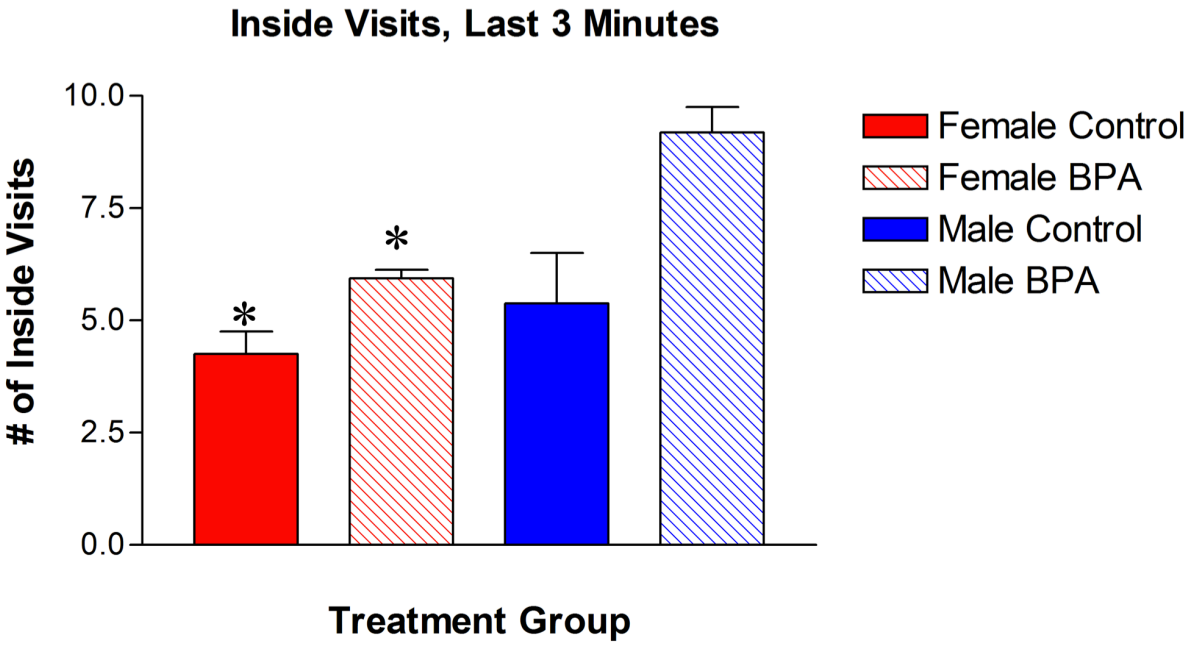
Figure 2

a.



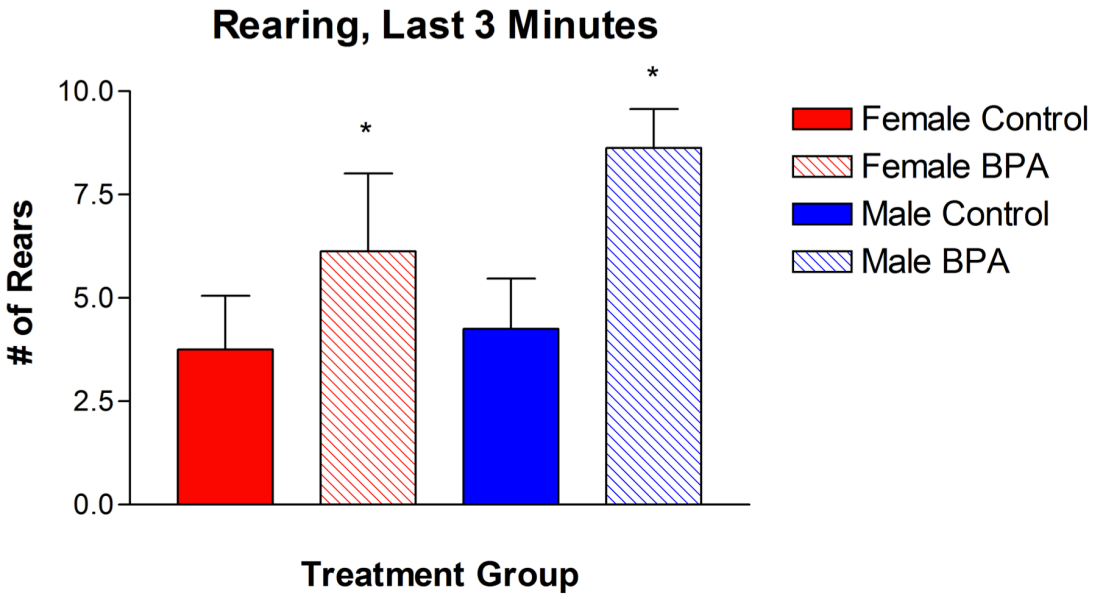
Open field, inside sector visits. A treatment effect and sex difference were observed in the first three minutes of open field, inside sector visits. BPA treated subjects had significantly more inside sector visits (8 m1.35) with respect to control (5.19 m0.95) and males visited inside sectors significantly more (8.44 m1.4) than females (4.75 m1.01) . Treatment $F(1,49)= 7.38$, $P<0.05$. Sex $F(1,49)= 4.29$, $P<0.05$

b.



Open field, inside sector visits. A sex difference were observed in the last three minutes of open field, inside sector visits. Males visited inside sectors significantly more (8.125 m1.347) than females (5.25 m0.952) . $F(1,49)= 4.55$, $P<0.05$.

Figure 3

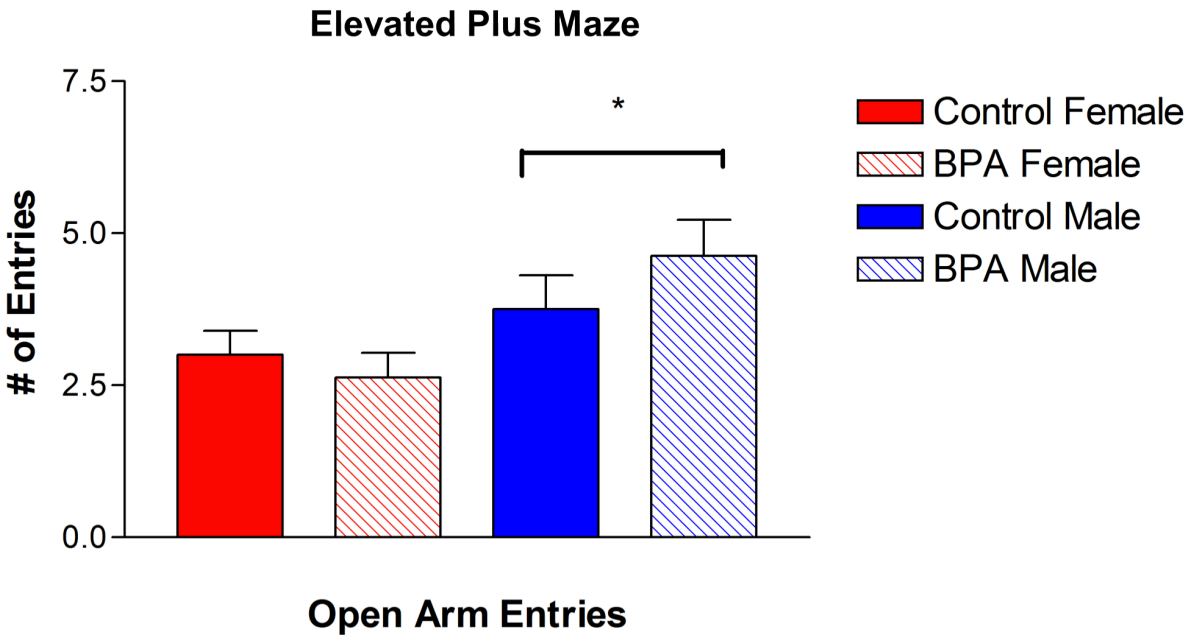


Open field, rearing. A treatment effect was observed in rearing behavior. BPA treated subjects had significantly more rears (7.375 m1.38) with respect to control (4 m0.979). $F(1,49)= 5.94$, $P<0.05$.

Elevated Plus Maze (Figure 4)

No BPA related changes in anxiety were observed. BPA treated rats did not have a significant difference in open arm entries, or time in open arms, compared with controls. A sex difference was observed in open arm entries. Males, 4.19 (SEM=0.56), had a significantly greater number of open arm entries compared to females, 2.81 (SEM=0.399), $P<0.05$. Although males showed slightly more overall arm entries than females, no significant overall activity difference was observed in elevated plus maze.

Figure 4

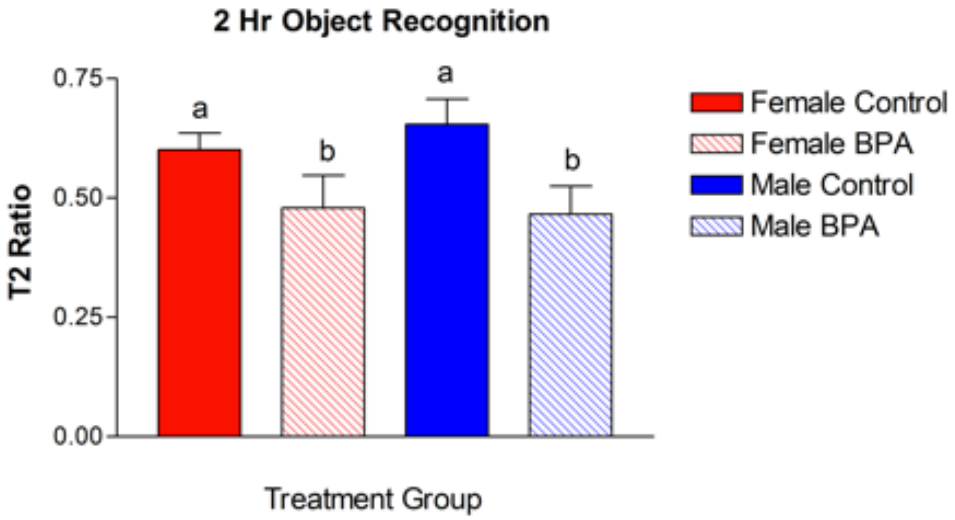


Elevated plus maze, open arm entries. A sex difference was observed in elevated plus maze. Males had significantly more open arm entries (4.18 m0.40) with respect to female (2.81 m0.56). $F(1,49)= 5.94$, $P<0.05$.

Object Recognition (Figure 5)

A treatment effect was observed in object recognition task performance. Male and female BPA treated subjects (0.47, SEM=0.03) had a significantly lower exploration ratio with respect to control subjects (0.628, SEM=0.05) in the 2-hour object recognition task, $P<0.01$. BPA subjects as a group explored the old object more frequently than the new object (ratio> 0.5). No significant differences were observed in the testing trial (T1). A slight sex difference was observed, however this was not significant. No significant differences were observed in the 4-hour object recognition task (data not shown).

Figure 5

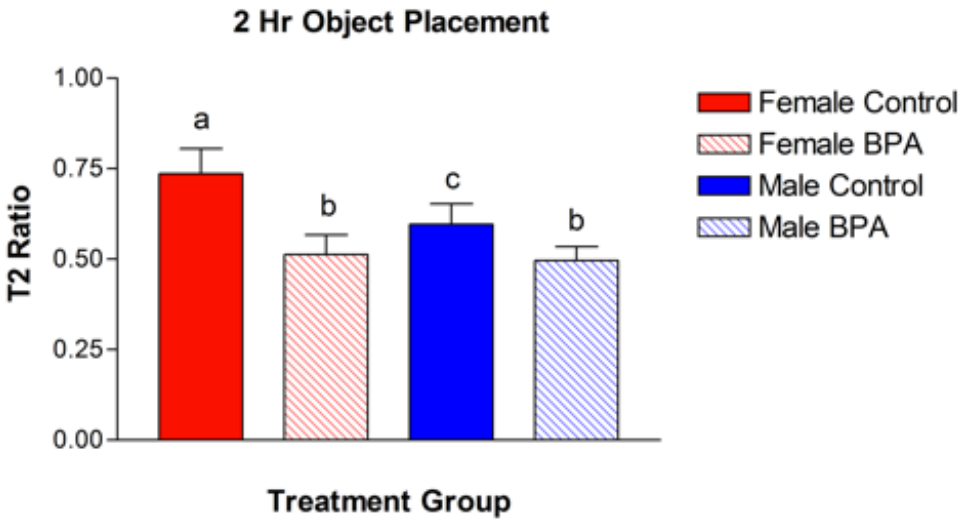


Two-hour object recognition. A treatment effect was observed in object recognition task performance. BPA treated subjects had a significantly lower exploration ratio (0.472m 0.03) with respect to control (0.628 m 0.05). $F(1,49)= 8.22$, $P<0.01$

Object Placement (Figure 6)

A treatment effect was observed in object placement task performance. Male and female BPA treated subjects had significantly lower exploration ratios, 0.505 (SEM=0.056), with respect to control subjects, 0.666 (SEM=0.040), in the 2-hour object placement task, $P<0.01$. No significant differences were observed in the testing trial (T1). No significant sex differences were observed in object placement task. No significant differences were observed in 4-hour object placement task (data not shown).

Figure 6



Two-hour object placement. A treatment effect was observed in object placement task performance. BPA treated subjects had significantly lower exploration ratios (0.505m 0.056) with respect to control (0.666m 0.040). $F(1,49)= 8.06$, $P<0.01$.

V. Discussion- Experiment 1- Prenatal BPA Exposure

Our initial experiment investigated the physiological, behavioral, and cognitive effects of prenatal BPA exposure. We first investigated the physiological effects of BPA exposure. We hypothesized that females, having less circulating estrogen during this developmental period, would have higher sensitivity to BPA. George, et al, observed increased body weight at adulthood in female rodents (2003), and increases in body weight of pups exposed in utero to BPA, at birth, and persisting through adulthood for females and to PND 54 in males, has also been observed (Rubin, et al, 2001).

Our data shows that prenatal exposure to BPA has significant physiological effects in both male and female rats. Exposure to BPA during the end of gestation is associated with severe decreases in juvenile body weight. Prenatal BPA exposure is also associated with sexually dimorphic alterations in adult weight. Male BPA subjects have significantly higher body weight at adulthood than their control counterparts. Overweight conditions were not observed in BPA females. These findings suggest that BPA exposure may interfere with weight set-point, and may contribute to underweight and overweight conditions at different stages of life. Existing data (Rubin, et al, 2001) is not consistent with our finding of decreased juvenile body weight in both sexes and increased body weight in adult males as a result of BPA exposure. The BPA dose was considerably higher in the previous study (0.1 and 1.2 mg/kg), and was administered for a longer time period (gestational day 6 to weaning). Our data suggests that body weight set point is affected at lower doses and shorter intervals of BPA exposure.

Behaviorally and cognitively, we hypothesized that females would be disproportionally affected by BPA exposure, specifically, that they would experience significant behavioral changes and cognitive deficits as a result of BPA exposure, specifically, increased locomotion, cognitive deficits and increased anxiety. In males, we hypothesized an increase in locomotor activity and decreased anxiety. Prenatal exposure to BPA induced behavioral changes in both sexes. Both sexes had increased locomotor activity, which is consistent our hypothesis, and with previous findings (Masuo, et al, 2004).

Anxiety was not altered by BPA in our study. Findings of previous studies are mixed. The existing literature suggests that effects of BPA on anxiety behaviors are sexually dimorphic. Ryan and Vandenbergh found that female mice exposed daily to bisphenol A in prenatal and early postnatal life (2 or 200 micrograms BPA) exhibited increased anxiety on elevated plus maze in a dose dependent fashion (2006). Farabollini, et al. observed decreased anxiety in male rats exposed to an environmentally relevant dosage of BPA during pre and early postnatal life (1999). Conversely, in a study by Fujimoto, et al, no change was observed in elevated plus maze performance in male or female rats administered 15 micrograms of BPA daily during gestation only (2008). It is plausible that the longer duration of exposure and higher dosage of BPA in the Ryan and Vandenbergh, and Farrabollini studies may have contributed to this result.

Both male and female BPA treated subjects had significant performance changes in both visual and spatial memory tasks. Exploratory ratios in object placement and object recognition tasks

were at or below .50, meaning that the BPA treated subjects explored the novel object/ location equally or less than the old object/location. Control groups of both sexes favored the novel location and object. We found no changes in anxiety behavior, therefore, we cannot attribute this difference in performance to anxiety. Object placement and recognition tasks are designed to measure spatial and visual memory (Luine V, Jacome L, MacLusky N, 2003). It appears that BPA subjects did not distinguish the old from the novel object/location, an indication of memory deficits. Impaired memory task performance suggests cognitive impairment in BPA subjects. Existing data on the effects of prenatal BPA exposure on cognition, specifically learning and memory, is sparse. In a previous study, male pups exposed to 0.1 and 50 mg/l of BPA in perinatal life had impaired performance in Morris Water Maze (Xu, et al, 2007). A study by Miyagawa, et al, found memory impairments (via step-through avoidance test) in male rats exposed perinatally to BPA (2007). The existing studies are primarily based on the effects in males only, and have much longer windows of exposure (gestation through weaning), and higher doses than our current study. In our study, the cognitive effects of prenatal exposure to BPA were not sexually dimorphic, as we hypothesized. Both sexes were significantly affected in the same behavioral areas (locomotor, visual and spatial memory), and unaffected in the same areas (anxiety). Thus, we speculate that BPA may alter more than estrogen-dependent effects during development. Such effects might be on thyroid action, as it has been shown that BPA is a thyroid receptor (T3) antagonist (Heimeier, et al, 2009). During perinatal life, thyroid hormone is important in brain development, and exposure to BPA can disrupt normal thyroid-mediated gene transcription (Zoeller, et al, 2005).

VI. **Experiment 2: Effects of Postnatal BPA Exposure**

In a previous study, we observed significant changes in locomotion as well as cognitive impairment in males and females exposed to 100 μg/kg of BPA during the last week of gestation (GD 16 to parturition). We observed low juvenile weight in both sexes and overweight in male pups administered 100 μg/kg of BPA during gestation. As previously stated, the time period from gestational day 18 to postnatal day 7 in rats is analogous to the period of sexual differentiation of the brain in humans, which occurs mid-gestation (MacLusky & Naftolin, 1981). During this period, BPA crosses the placenta, exposing human fetuses directly through the bloodstream. Exposure to BPA may continue throughout neonatal life through breast milk or exposure to BPA-containing plastics. This study will focus on pups' exposure to BPA via direct measured administration (subcutaneous injection). The aim of this project is to investigate the effects of exposure to bisphenol A— two doses, 60 μg and 100 μg/kg body weight---during the first week post-birth (parturition to PND 6). We will investigate whether exposure to this compound induces neurochemical changes in the brains of male and female rat pups at adulthood. We will also determine if disruption of these brain regions is associated with changes in anxiety, locomotion, and memory.

Research Design and Methods

Breeding

Adult, Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. and allowed to acclimate to environment before mating. Mating was conducted at Hunter College Animal facility in order to eliminate maternal stress during shipping as previously reported by the laboratory (Bowman, et al, 2006). Breeder and offspring animals were raised on standard rat chow. Eight, 3 month old females received mating, and four, 3 month old, males, were used as studs. Females were housed separately and received vaginal smears each morning around 10 AM to determine estrus cycle day. If a female was in proestrus, a male was housed with the female overnight (beginning around 3-4 PM) in order for mating and pregnancy to occur. The male was removed in the morning, cage checked for vaginal plugs, and the female received a vaginal smear to determine presence of sperm. Females continued to receive smears and mating until successful mating/pregnancy of at least 5-6 females occurred (This is based on the need for 60 offspring from mothers with a reported mean litter size of 12-16 pups/litter. So, even if each dam has only 10 pups, lower than average, then 6 mothers x10 pups=60 total pups). After some delays in the mating process due to a problem with a stud male, five of the eight females became pregnant.

Treatment Groups and Injections

Starting at day 22 of pregnancy, dams were checked for delivery of pups at 10 AM and at 2, 4 and 6 PM (animal facility personnel checked animals at 9 AM on weekends). A total of 60 pups was sought (10 per sex/group x 3 groups). We obtained 5 litters. All litters were culled to 10. More pups were born than anticipated, and were euthanized on day 1. The surviving pups were tail marked as to treatment and sex. Controls received one tail ring, high dose two tail rings, low

dose pups three tail rings. On postnatal day 2, 4 and 6, pups received a single subcutaneous injection of 1% ethanol/saline (control), 60 μg/kg BPA (low dose), or 100 μg/kg BPA (high dose) in 1% ethanol/saline vehicle. Treated pups were distributed among the five litters. Pups were weaned at 21 days and group housed, by sex, in the Hunter College Animal Facility. As they grew, the number of pups/cage were gradually decreased until at adulthood, two adults are present in each cage. Three cohorts (16, 16, 18) were established with rats from different treatment groups, and counterbalanced for sex, treatment, and litter. Behavioral testing began on postnatal day 60.

Physiological measures-

Rats were weighed daily during injections, and biweekly until sacrifice.

Female rats were inspected daily for evidence of vaginal opening beginning on PND 21.

Behavior-

At two months of age, behavioral testing began in 50 rats. Open field behavior (locomotor activity), memory assessment by object recognition (visual) and object placement (spatial), as well as elevated plus maze (anxiety) were conducted. Behavior was run in 3 cohorts with approximately 16 rats/cohort. Cohort assignment depended on date of birth (earlier date of birth tested first). The performance of experimental groups on the task was compared with performances of the control group of their same sex, and across treatment groups. Behavioral analyses were identical to the previous experiment (please see experimental procedures, above).

Behavior was analyzed by two-way ANOVA to determine sex differences and treatment effects (sex x treatment). Post hoc testing was by LSD test.

Two weeks following behavioral task completion, the brains of control and experimental groups were removed, hand-sectioned coronally using a single-edged razor blade, and analyzed via HPLC with electrochemical detection to measure monoamine levels in specific brain regions. Monoamine data was analyzed by two-way ANOVA (sex x treatment).

Following sacrifice, testes, prostate, uteri and ovaries were harvested and weighed.

Neuro*chemical Measurements*

HPLC-

Following completion of the behavioral tests, rats were sacrificed via rapid decapitation, and the brains rapidly frozen and stored at –70 ˚ C. Hand sectioning with a razor blade was performed to obtain coronal serial sections of the brain of approximately 2mm thick. Once sectioned, slices were frozen to a slide and kept at –70 ˚ C. Using a 500μm-diameter cannula, tissue samples from various brain regions were obtained from the frozen section under a dissecting microscope with the stage maintained at approximately –11 ˚C and placed in a 1.5ml Eppendorf tubes. Between two to eight punches were taken, dependent upon the area and neurotransmitter being measured. Monoamines and metabolites neurotransmitters levels were measured by dissolving the punches in 60μl of sodium acetate buffer, pH 5.0, and a process of freezing and thawing was used to disrupt cellular structures and release cellular components including neurotransmitter of interest.

α-A-Methyl-dopamine was added as an internal standard and samples were centrifuged at 12000 r.m.p. for 12 minutes. The supernatant was removed and the pellet was re-suspended in 100 or 200µl 2.0N NaOH, depending on the amount of punches, for protein analysis using Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA, USA).

High-performance liquid chromatography (HPLC), with electrochemical analysis was used to quantify neurotransmitter levels using methods previously utilized in the lab (Bowman, et al, 2002). The 40µl supernatant was used in the detection of monoamines, including dopamine and its metabolites, 3-4-dihydroxyphenylalanine (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and its metabolite 3-Methoxy-4-Hydroxyphenylglycol (MHPG); and serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA). Monoamines were measured in a Waters Associates chromatographic system (Waters 2690) consisting of an alliance module containing an automated refrigerated, injector, pump, Symmetry C_{18} 5µm 4.6 X 150mm reverse-phase column (Novapak three micron), and an ESA Coulochem III detector (0.45V potential). The mobile phase, described elsewhere (Bowman, et al, 2002), contained 3% acetonitrile and peak sharpness was increased by the addition of 100% methanol (99.5% mobile: 0.5% methanol).

Millennium software (Waters Associates) was used to run the chromatography system and concentrations of transmitters and metabolites were calculated by reference to standards using peak integration. Monoamines levels were measured in brain areas, including the frontal cortex,

CA1 and CA3 of hippocampus, striatum, basolateral amygdala, and medial preoptic nucleus. Results were analyzed by two-way ANOVA (sex x treatment). Post hoc testing by LSD test.

VII. Results

Physiological Measures

Juvenile Body Weight (Figure 7a,b)

In the time period from PND 6 to PND 17, there was no significant difference in the body weight of female or male BPA treated subjects with respect to control subjects.

Figure 7a

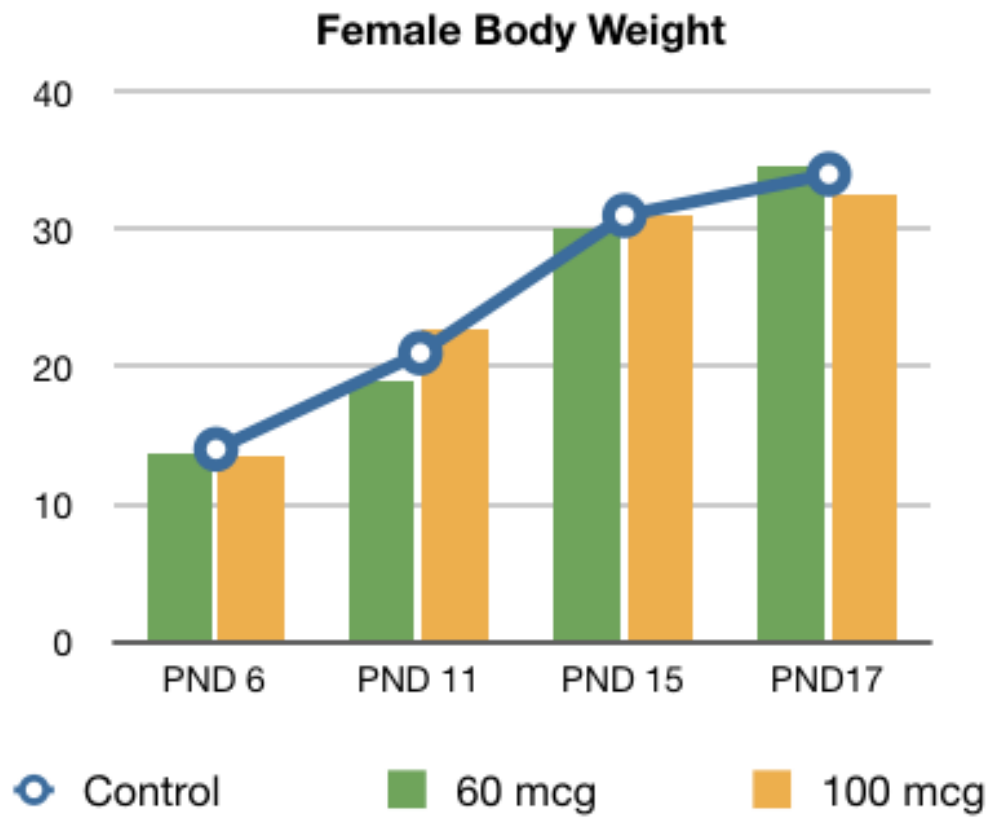
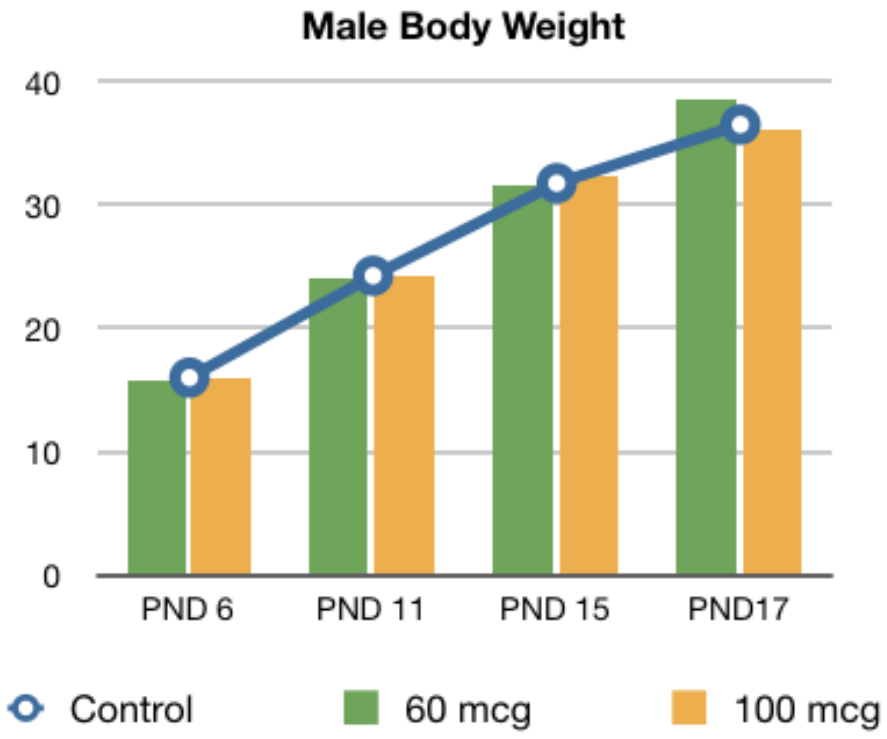

Female Body Weight
40
30
20
10
0
PND 6
PND 11
PND 15
PND17
Control
60 mcg
100 mcg

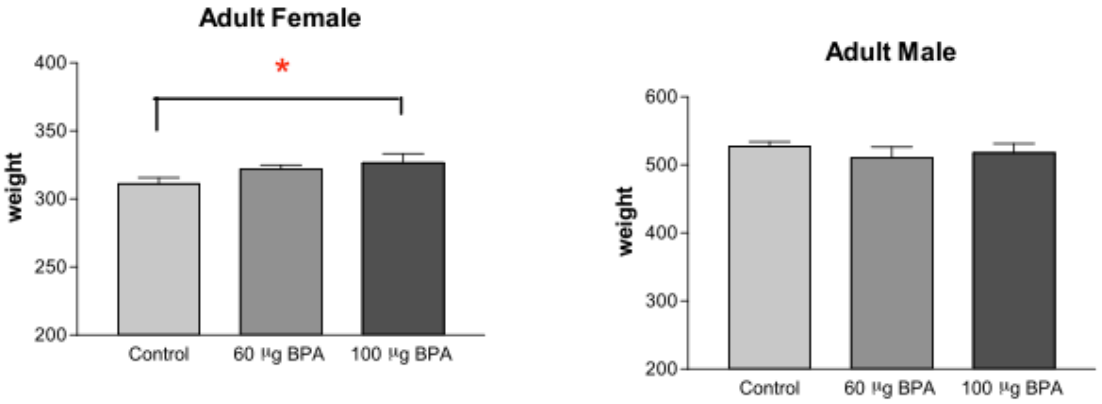
Figure 7b


Male Body Weight
40
30
20
10
0
PND 6
PND 11
PND 15
PND17
Control
60 mcg
100 mcg

Adult Body Weight- (Figure 7c,d)

At date of sacrifice, there was no significant weight difference among male subjects. A significant increase in body weight was observed in 100 μg/kg dose females, 311.5 (SEM=4.458) with respect to control, 326.4 (SEM=6.845), $P< 0.05$.

Figure 7 (c, d)



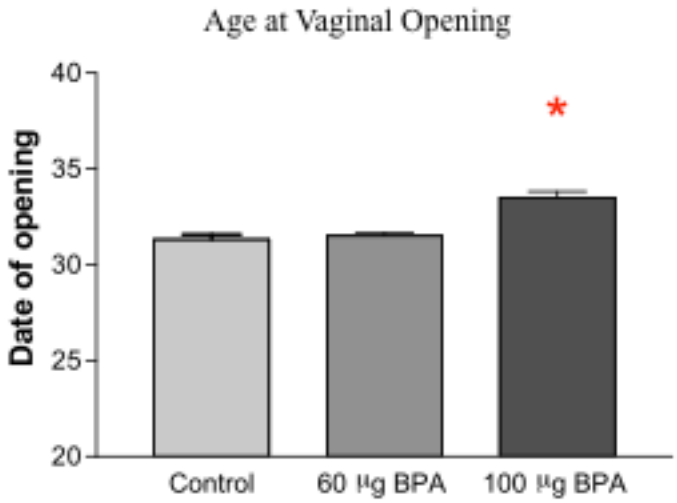
Body weight at adulthood. 100 microgram treated females had significantly higher body weight with respect to control females. $F(2,23)=2.5$ ($^*P<0.05$)

Age at Vaginal Opening/Genital Tract Morphology- (Figure 8, Table 2)

Vaginal opening was significantly delayed in 100 μg/kg female subjects, occurring on postnatal day 33.5 (SEM=0.327) with respect to 60 μg/kg, PND 31.5 (SEM=0.189), and control, PND 31.3 (SEM=0.236) groups, $P<0.05$.

No significant differences were observed in wet weight of testis, prostate, uterus and ovary.

Figure 8



Age at vaginal opening. Age at vaginal opening was significantly delayed in 100 microgram treated subjects. $F(2,23)=21.85$ ($^*P<0.05$)

Table 2

Reproductive Organ Weights

	Control	60 mcg BPA	100 mcg BPA
Female			
Uterus (g)	1.078 (.210)	1.269 (.315)	1.031 (.223)
Ovaries (g)	0.389 (0.046)	0.381 (0.067)	0.481 (0.069)
Male			
Testes (g)	4.240 (0.138)	4.036 (0.165)	4.081 (0.154)
Prostate (g)	0.928 (0.094)	0.942 (0.116)	0.886 (0.107)

Behavior

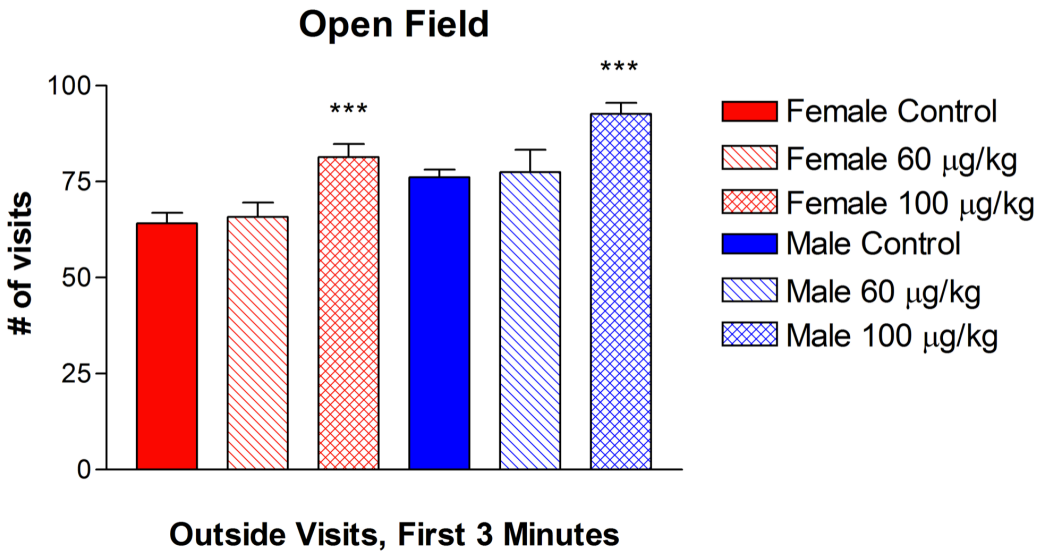
Open Field- (Figure 9, 10, 11)

A treatment effect was observed in locomotor activity. 100 μg/kg BPA treated subjects, male and female, had significantly more outside visits, wall climbs, and rearing than control and 60 μg/kg dose subjects. In the first three minutes of open field, 100 μg subjects had significantly more outside sector visits, 87 (SEM=2.902) than 60 μg, 71.2 (SEM=5.842) and control, 70.42 (SEM=2.002), $P<0.001$. A significant difference in outside sector visits was also observed between 60 μg and control subjects. Males had a significantly greater number of outside sector visits in the first three minutes of open field, 81.76 (SEM=1.965) to 70.16 (SEM=1.649), $P<0.001$. This difference was not observed in females.

100 μg subjects, males and females, had significantly more rears, 9.3 (SEM=0.44), than 60 μg, 5.4 (SEM=0.64) and control, 5.7 (SEM=0.43) subjects, $P<0.001$. A sex difference in rearing behavior was also observed, with males having significantly more rears, 7.6 (SEM=0.44), than females, 6 (SEM=0.43), $P<0.05$.

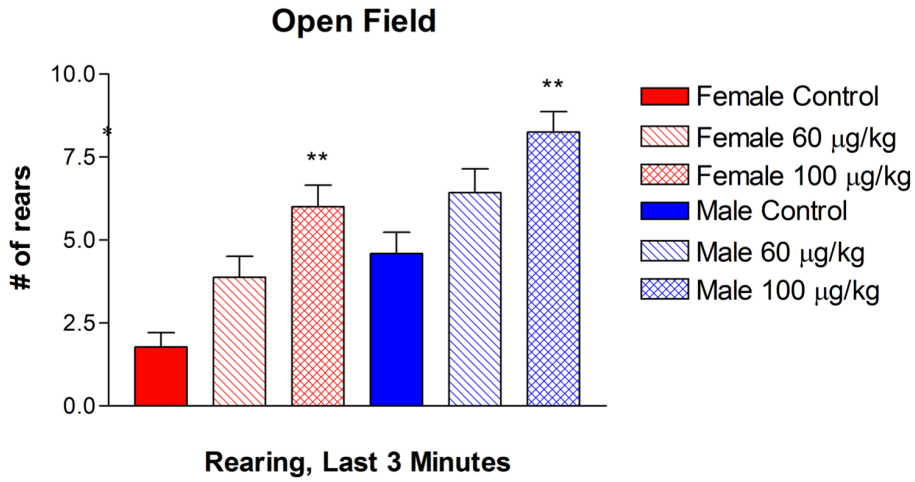
In the first three minutes of open field, 100 μg subjects had significantly more wall climbs, 7.1 (SEM=0.45) than 60 μg, 5.1 (SEM=0.46) and control, 3.3 (SEM=0.41), $P<0.001$. A significant difference in wall climbs was also observed between 60 μg and control subjects. Males had a significantly greater number of wall climbs in the first three minutes of open field, 6.3 (SEM=0.36) to 3.8 (SEM=0.6), $P<0.001$. This difference was not observed in females.

Figure 9



Open field, outside sector visits. A treatment effect was observed in number of outside visits. 100 μg/kg subjects had significantly more outside sector visits (87 m2.902) with respect to control (70.42m2.002) and 60 μg/kg dose subjects (71.2 m5.842). $F(2,48)=14.93$, $P<0.001$. An overall sex difference was also observed, Female (70.16 m1.649), Male (81.76 m1.965). $F(1,49)=17.33$, $P<0.001$.

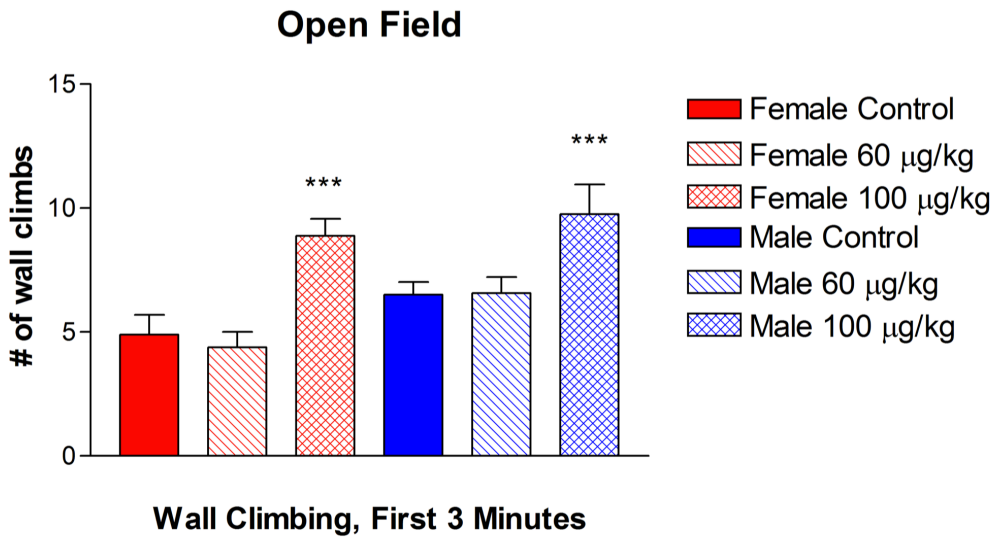
Figure 10



*

Open field rearing behavior. A treatment effect was observed in number of rears. 100 μg/kg subjects had significantly more rearing episodes (9.312 m0.438) with respect to control (5.736m0.43) and 60 μg/kg dose subjects (5.4 m0.639). $F(2, 48)=15.34$, $P<0.001$. A sex difference was also observed in rearing behavior. Males reared significantly more times (7.56m0.44) than females (6m0.43). $F(1, 49)=6.03$, $P<0.05$

Figure 11



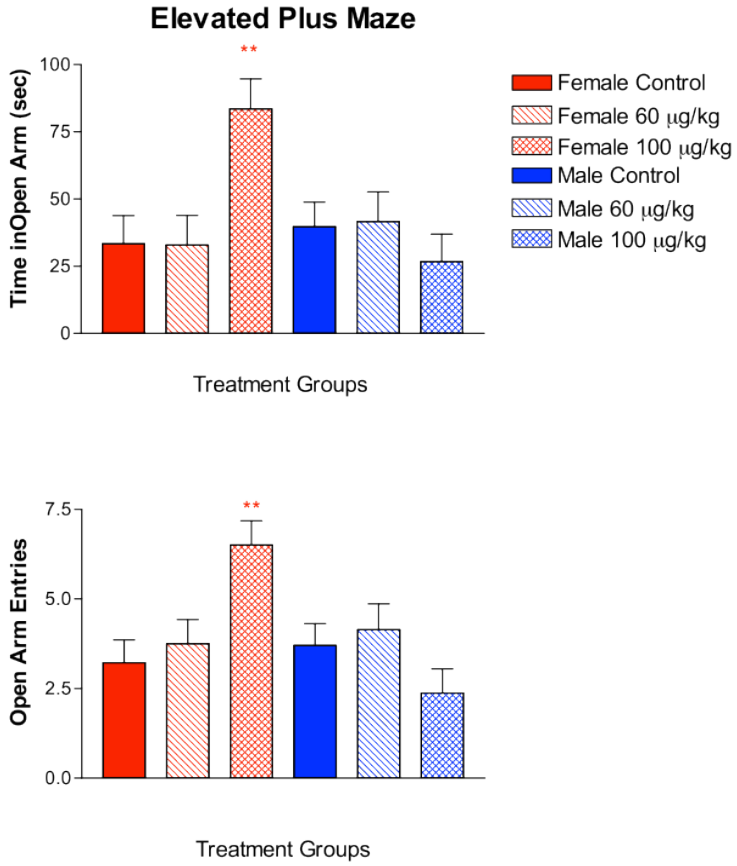
Open field wall climbing. A treatment effect was observed in number of wall climbs. 100 μg/kg subjects had significantly more wall climbs (7.125 ±0.446) with respect to control (3.263±0.409) and 60 μg/kg dose subjects (5.067±0.461). A significant difference between 100 and 60 μg/kg dose groups was also observed. $F(2, 48)=20.03$, $P<0.001$. An overall sex difference was also observed. Males had significantly greater number of wall climbs (6.28 ±0.357) when compared to females (3.8 ±0.594). $F(1, 49)=25.06$, $P<0.001$

Elevated Plus Maze- (Figure 12a,b)

No treatment or sex effects were observed in elevated plus maze performance. A sex-treatment interaction was observed in elevated plus maze performance.

High dose females spent significantly more time in open arms, 83.6 seconds (SEM=11.13), versus 34.2 seconds (SEM=10.69), $P<0.01$. High dose females had a significantly greater number of open arm entries, 6.5 entries (SEM=0.68), with respect to all treatment groups, 3.4 entries (SEM=0.64), $P<0.01$. This effect was not observed in males.

Figure 12a,b



Elevated plus maze performance. A sex-treatment interaction was observed in elevated plus maze performance. High dose females spent significantly more time in open arms (83.57s m11.13) with respect to all treatment groups $F(5,45)=6.24$, $P<0.01$. High dose females had a significantly greater number of open arm entries (6.5m0.68) with respect to all treatment groups. $F(5,45)=7.76$, $P<0.01$.

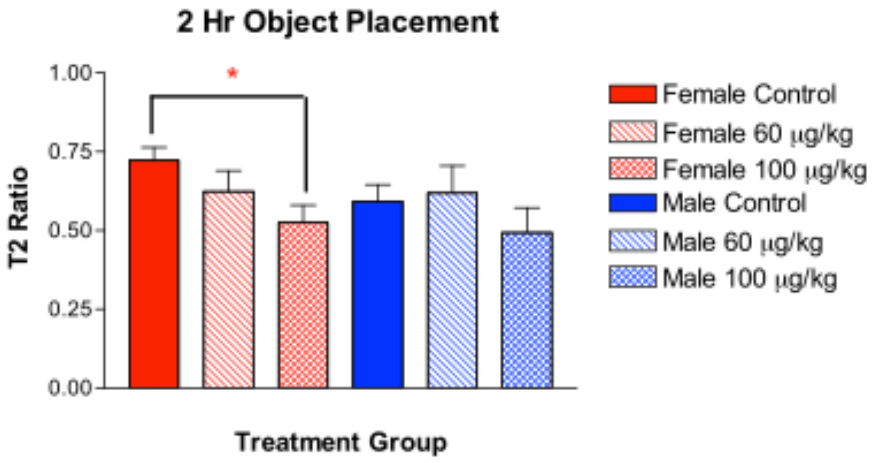
Object Recognition- (data not shown)

No treatment-related significant difference in performance was observed in object recognition task at 2 and 4 hour intertrial delay. No sex difference or sex-treatment interaction differences were observed.

Object Placement- (Figure 13)

No significant differences were observed in the 2 or 4 hour object placement task when analyzed with two-way ANOVA. When subjects were analyzed separately by sex, a treatment effect was observed in 2-hour object placement task. High dose females had a significantly lower exploratory ratio, 0.5 (SEM=0.06), compared to control females ratio of 0.7 (SEM=0.04), $P<0.05$. This effect was not observed in males. There was no significant difference observed in T1 (sample trial) exploration.

Figure 13



Two-hour object placement. When analyzed across sex and treatment parameters, no significant differences were observed in the object placement task. When subjects were analyzed separately by sex, a treatment effect was observed. High dose females had a significantly lower exploratory ratio (0.5248 m 0.06) with respect to control females (0.7219 m 0.04). $F(2,23)= 3.44$, $P<0.05$.

Neurochemical Measurements

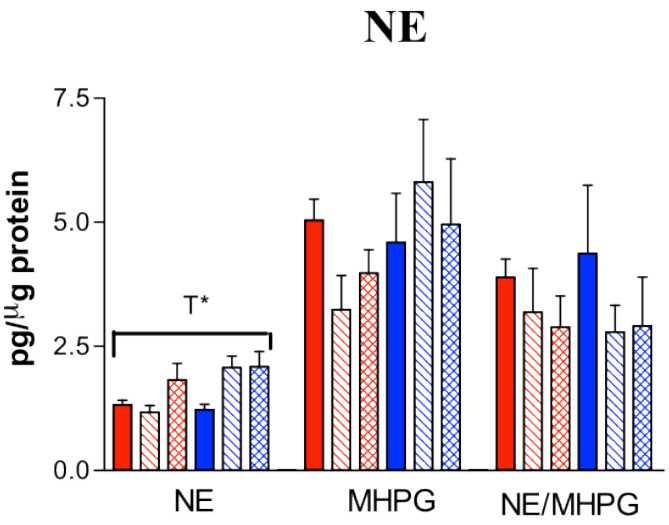
Main effects are listed first, and then any sex-treatment interactions. Please refer to figure legends for detailed interaction information.

CA1of Hippocampus (Figure 14 a,b,c)

In NE concentration, an overall treatment effect was observed. 100 μg dose subjects had significantly greater concentration of NE (1.94) with respect to control (1.94), $P<0.05$. 100 μg subjects had an approximately 60% increase in NE levels when compared to control levels. When viewed separately by sex, in males, both 60 and 100 μg dose subjects experienced an increase in NE concentration. In females, only the high dose produced an effect. There were no significant differences in monoamine concentration or turnover ratios in DA, 5HT, or their metabolites.

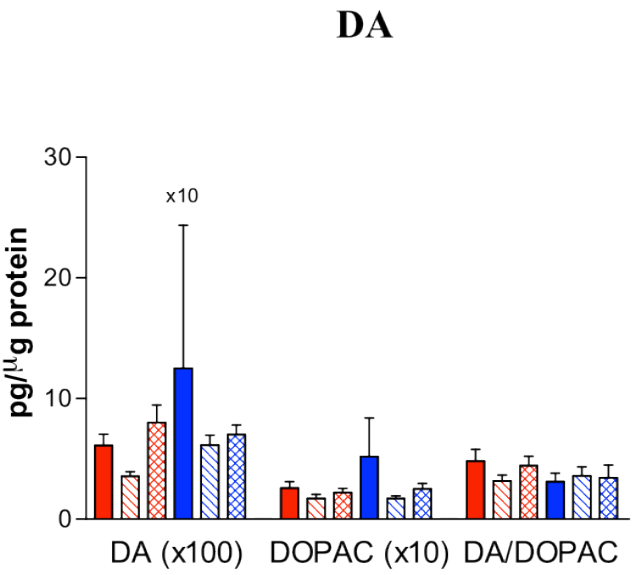
Figure 14 a

CA1



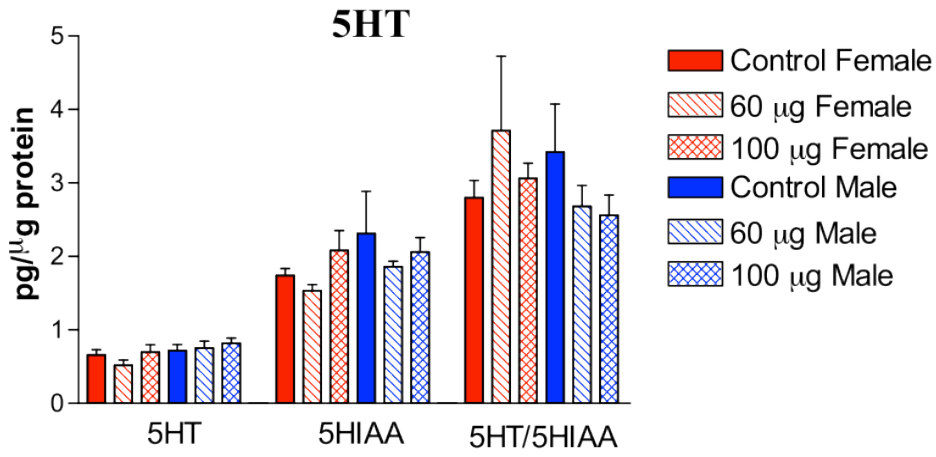
Concentration and turnover ratio of norepinephrine and its metabolite, MHPG, in CA1 of hippocampus. NE: A treatment effect was observed in high dose subjects. High dose subjects has significantly higher levels of NE with respect to control. NE Treatment- $F(2,48)= 4.49$, $P<0.05$.

Figure 14 b



Concentration and turnover ratio of dopamine and its metabolite, DOPAC, in CA1 of hippocampus.

Figure 14 c



Concentration and turnover ratio of serotonin and its metabolite, 5HIAA, in CA1 of hippocampus.

CA3 of Hippocampus (Figure 15 a,b,c)

NE/MHPG: In NE concentration, a treatment effect was observed. 100 μg BPA subjects had decreased NE concentration, and greatly increased MHPG concentration, approximately two-fold higher than 60 μg and control subjects. A sex difference was observed, males had a significantly higher concentration of NE and MPHG with respect to females. Activity in the NE system was also affected by BPA. 100 μg dose subjects had an approximately three-fold increase in turnover of NE to metabolite MHPG in males and females.

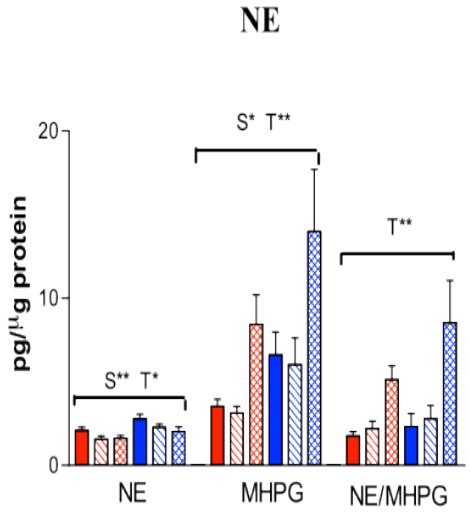
DA/DOPAC: 100 μg dose treatment increased DA concentration by 60% in males and females. A sex difference was observed in DOPAC concentration. Males have significantly higher DOPAC concentration with respect to control females.

In a sex-treatment interaction, the sex difference in DOPAC concentration was abolished in low dose males.

In 5HT and 5HIAA, a sex difference was observed, treatment with BPA had no significant effects on serotonin levels. Concentration of 5HT and 5HIAA is significantly higher in males. No sex difference was observed in 5HT/5HIAA turnover.

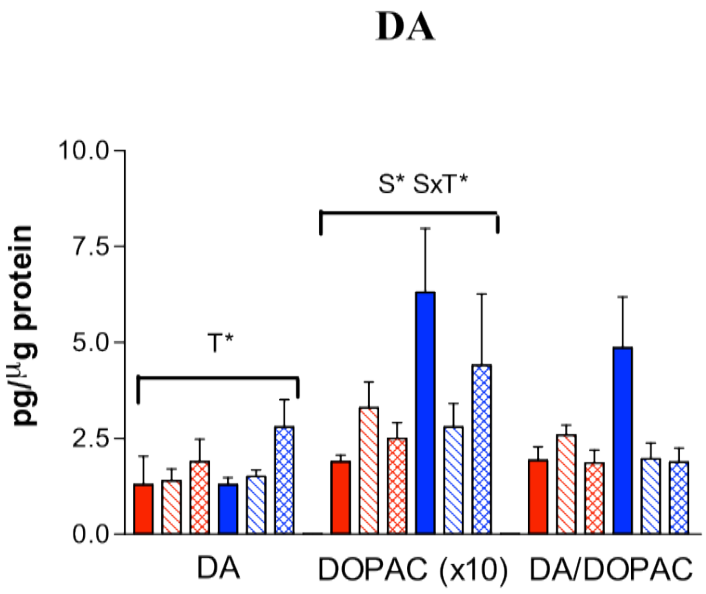
Figure 15 a

CA3



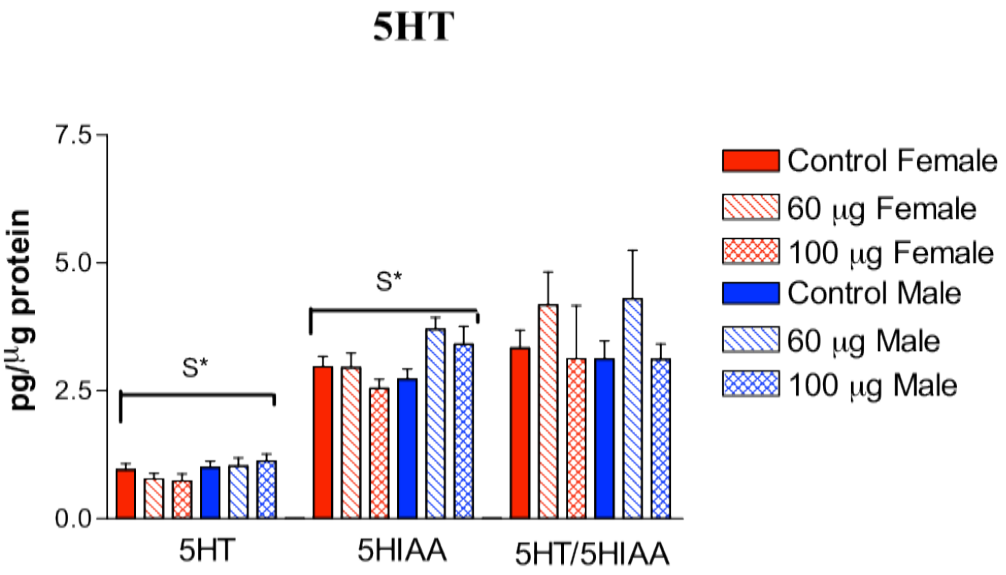
Concentration and turnover ratio of norepinephrine and its metabolite, MHPG, in CA3 of hippocampus. NE: A sex difference was observed in NE and MPHG concentration. Males had a higher concentration overall of these monoamines. NE Sex- $F(1,49)$: 8.09, $P<0.01$, MHPG Sex- $F(1,49)=5.27$, $P<0.05$. A treatment effect was observed in high dose subjects in NE concentration, which was decreased, Treatment- $F(2, 48)= 3.84$, $P<0.05$, MHPG concentration, which was increased, Treatment- $F(2,48)= 5.77$, $P<0.01$, and in the turnover of NE to metabolite MHPG. High dose treatment increased this turnover, NE/MHPG Treatment- $F(2,48)=7.48$, $P<0.01$.

Figure 15 b



Concentration and turnover ratio of dopamine and its metabolite, DOPAC, in CA3 of hippocampus. DA: A treatment effect was observed in DA concentration. High dose treatment significantly increased DA concentration, DA Treatment- $F(2,48)=7.48$, $P<0.05$. A sex difference was observed in DOPAC concentration. Males have higher concentration of DOPAC, DOPAC Sex- $F(1,49)=6.47$, $P<0.05$. A sex-treatment interaction was observed in low dose males' concentration of DOPAC, which was significantly lower with respect to control, Sex x Treatment- $F(5,45)=3.48$, $P<0.05$.

Figure 15 c



Concentration and turnover ratio of serotonin and its metabolite, 5HIAA, in CA3 of hippocampus.5HT: A sex difference was observed in 5HT and 5HIAA concentration, 5HT Sex- $F(1,49)=4.70$, $P<0.05$, 5HIAA Sex- $F(1,49)=5.87$, $P<0.05$. Males had higher overall concentration of the monoamine and its metabolite.

Basolateral Amygdala (Figure 16 a,b,c)

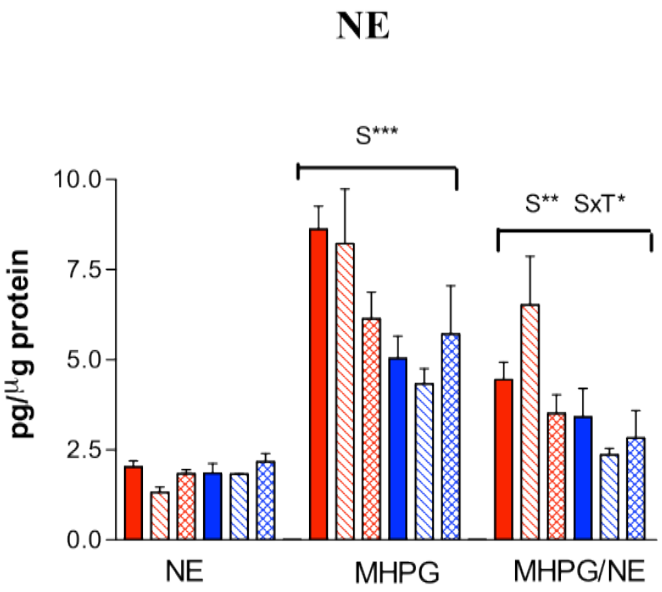
NE/MHPG: A sex difference was observed in MHPG concentration and in NE/MHPG turnover. Females have significantly higher MHPG concentration and NE/MHPG turnover ratio. A sex-treatment interaction was observed in NE activity. NE/MHPG turnover ratio was significantly higher in low dose females, nearly two-fold, with respect to all treatment groups.

DA/DOPAC: A main effect of BPA treatment was observed in DA concentration.100 μg subjects had a two-fold greater concentration of DA with respect to control, and a 70% increase with respect to 60 μg subjects. Sex/treatment interactions were observed in DA and DOPAC concentrations, and in DA/DOPAC turnover. 100 μg dose males and 60 μg dose females had over two-fold higher concentration of DA. DOPAC concentration was significantly less in low dose males. Lower DA/DOPAC turnover was observed in 60 μg females and 100 μg males.

5HT/5HIAA: A sex difference was observed in 5HIAA concentration. Females have a higher concentration overall (3.6 to 3.1, respectively). A sex-treatment interaction was observed in 5HT concentration, which was higher in 60 μg males; and 5HT/5HIAA turnover, higher in 60 μg females, lower in 60 μg males.

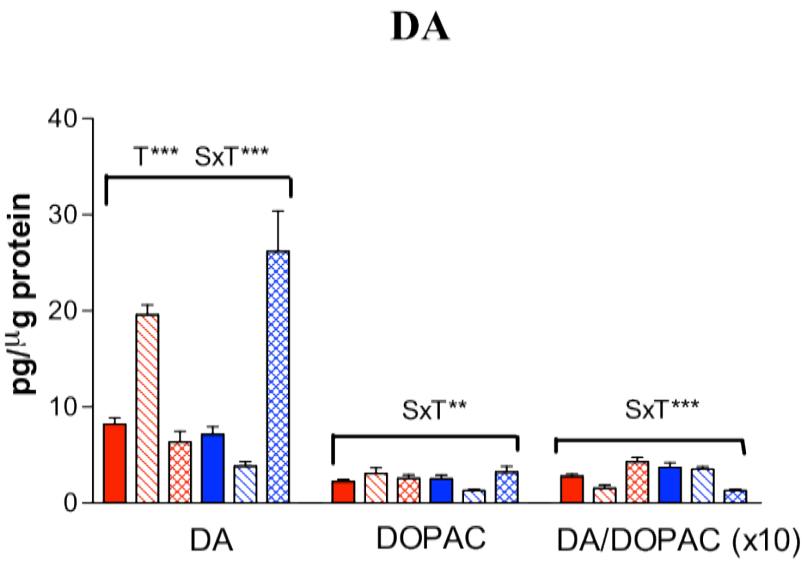
Figure 16 a

BLA



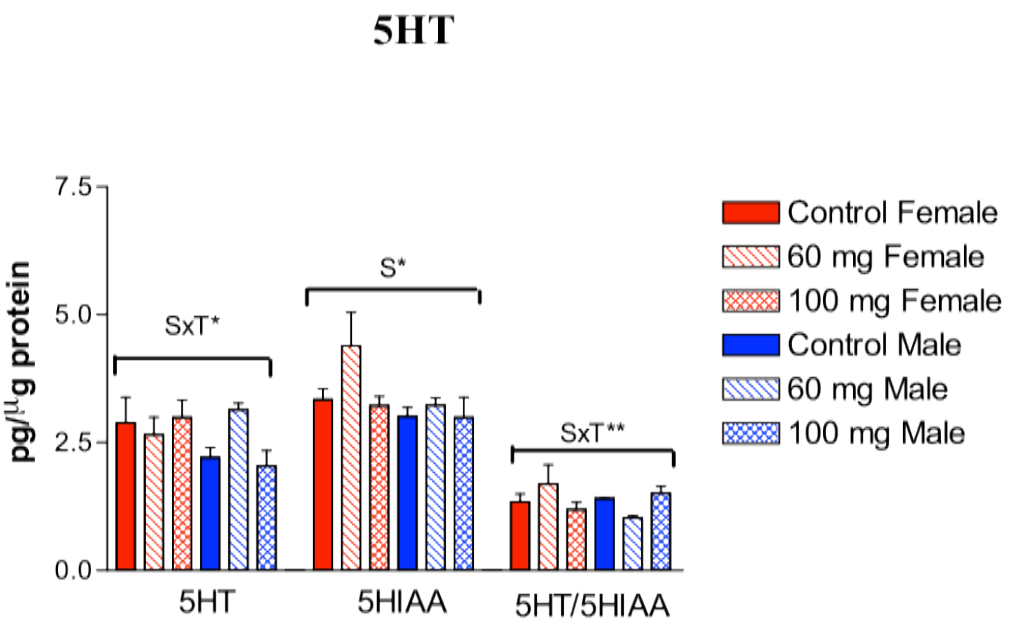
Concentration and turnover ratio of norepinephrine and its metabolite, MHPG, in basolateral amygdala. NE: A sex difference was observed in MHPG concentration, MHPG Sex- $F(1,49)=12.6$, $P<0.001$, and NE/MHPG turnover ratio, NE/MHPG Sex- $F(1,49)=10.93$, $P<0.01$, both of which were greater in females overall.
A sex-treatment interaction was observed in NE/MHPG turnover ratio, Sex x Treatment- $F(5,45)=3.48$, $P<0.05$. Treatment with low dose of BPA increased activity in females.

Figure 16 b



Concentration and turnover ratio of dopamine and its metabolite, DOPAC, in basolateral amygdala. DA: A treatment effect was observed in DA concentration, DA Treatment- $F(2,48)=10.86$, $P<0.001$, with increased concentration in high dose subjects. A sex-treatment interaction was observed in DA and DOPAC concentration, with a decrease concentration in low dose males, DA Sex x Treatment- $F(5,45)=45.25$, $P<0.001$, DOPAC Sex x Treatment- $F(5,45)=5.94$, $P<0.01$; and DA/DOPAC turnover, DA/DOPAC Sex x Treatment- $F(5,45)=21.46$, $P<0.001$, with decreases in both low dose females and high dose males.

Figure 16 c



Concentration and turnover ratio of serotonin and its metabolite, 5HIAA, in basolateral amygdala. 5HT: A sex difference was observed in 5HIAA concentration, 5HIAA Sex- $F(1,49)=5.02$, $P<0.05$, with higher levels in females.
A sex-treatment interaction was observed in 5HT concentration, 5HT Sex x Treatment- $F(5,45)=3.53$, $P<0.05$, and 5HT/5HIAA turnover, 5HT/5HIAA Sex x Treatment- $F(5,45)=7.64$, $P<0.01$, with effects seen in low dose males (5HT) and low dose males and females (turnover).

Frontal Cortex (Figure 17 a,b,c)

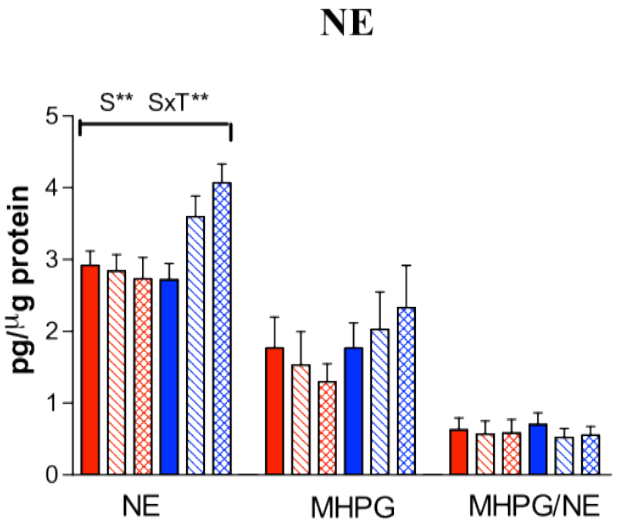
NE/MHPG: A sex difference was observed in concentration of NE. Males had a higher overall concentration of NE (3.32 to 2.83, females). A sex-treatment interaction was also observed. 60 μg and 100 μg dose males had increased concentration of NE, with respect to control males and all female groups.

DA/DOPAC: Sex differences were observed in DA and DOPAC concentrations, with two-fold higher concentration in males.

5HT/5HIAA: No significant differences were observed in concentration of 5HT, 5HIAA, or turnover ratio.

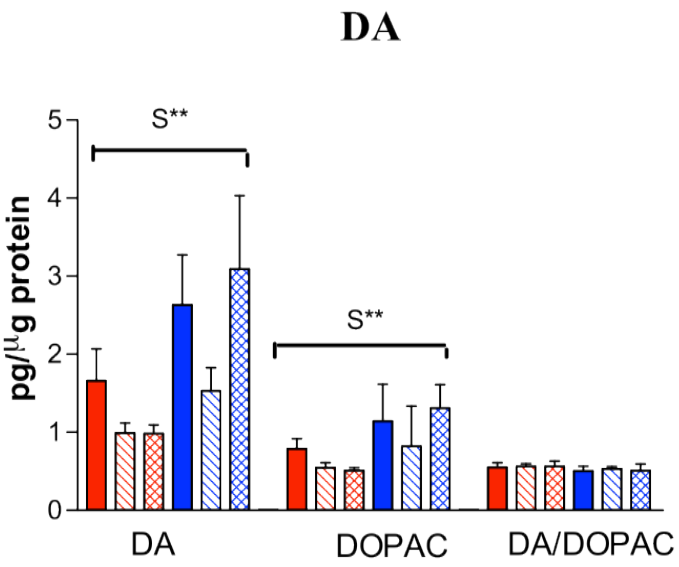
Figure 17 a

Frontal Cortex



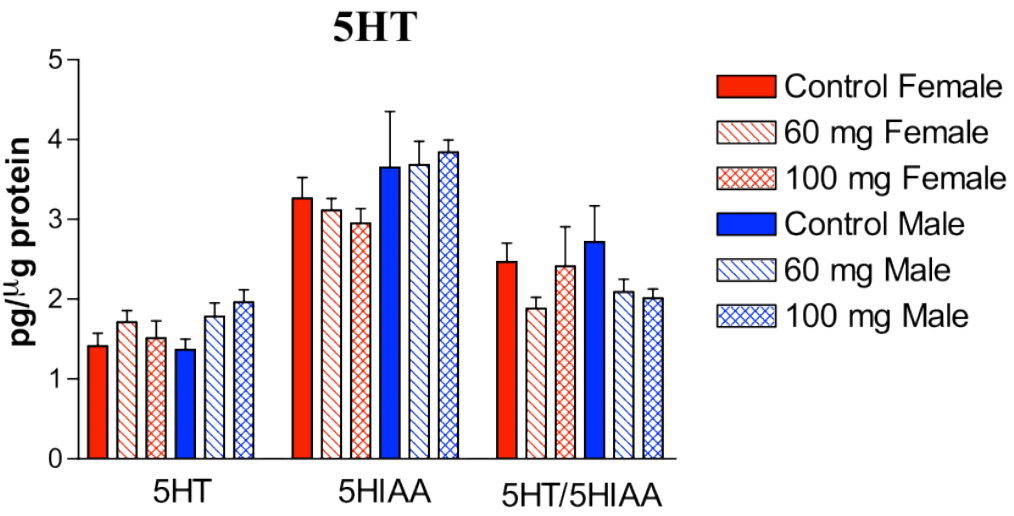
Concentration and turnover ratio of norepinephrine and its metabolite, MHPG, in frontal cortex. NE: A sex difference was observed in concentration of NE, NE Sex- $F(1,49)=7.10$, $P<0.01$, with higher concentration overall in males. A sex-treatment interaction was observed in concentration of NE, Sex x Treatment- $F(5,45)=5.22$, $P<0.01$, affecting low and high dose males.

Figure 17 b



Concentration and turnover ratio of dopamine and its metabolite, DOPAC, in frontal cortex. DA: Sex differences were observed in DA and DOPAC concentration, DA Sex- $F(1,49)=7.45$, $P<0.01$, DOPAC Sex- $F(1,49)=10.15$, $P<0.01$, with greater concentration in males.

Figure 17 c



Concentration and tunrover ratio of serotonin and its metabolite, 5HIAA, in frontal cortex.

MPOA (Figure 18 a,b,c)

NE/MHPG: A treatment effect was observed in NE concentration and NE/MHPG turnover ratio. NE concentration was significantly lower in 60 μg subjects, half of the level of 100 μg and control subjects, and turnover was increased two-fold in 60 μg subjects. A sex-treatment interaction was observed in concentration of NE (60 μg males—a 50% decrease); and in concentration of MHPG (100 μg females) and turnover ratio of NE/MHPG (60 μg males and females). An increase in MHPG concentration and increased turnover was observed in these groups.

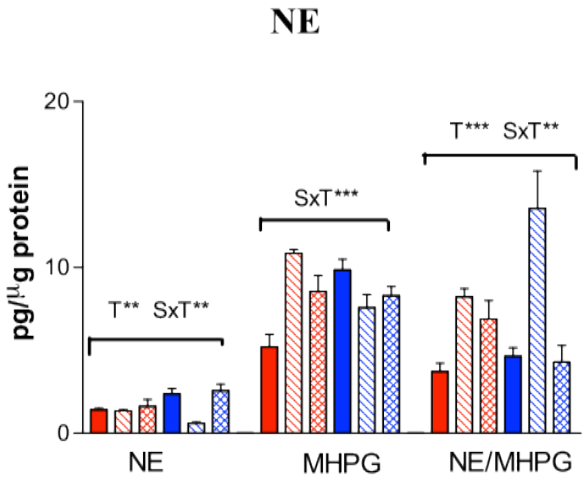
DA/DOPAC: A sex difference was observed in DA and DOPAC concentration. Males had an approximately two-fold higher concentration of DA, and higher concentration of DOPAC. A treatment effect was observed in DA/DOPAC turnover, 60 μg subjects had a significantly higher turnover ratio. 60 μg BPA treatment in females lowered DA concentration, and increased DA/ DOPAC turnover (sex/treatment interaction).

5HT/5HIAA: A treatment effect was observed in 5HT and 5HIAA concentration, which was significantly lower in 60 μg subjects.

A sex-treatment interaction was observed in 5HT concentration, with decreased concentration in 60 μg males. A main effect of sex difference was seen in 5HT/5HIAA turnover. Females had 80% higher 5HT turnover with respect to males. Sex/treatment interaction was also observed in 5HT/5HIAA turnover ratio, which was lowered in 60 μg females, and increased in 100 μg males.

Figure 18 a

MPOA



Concentration and turnover ratio of norepinephrine and its metabolite, MHPG, in medial preoptic area. NE: A treatment effect was observed in the NE concentration and NE/MHPG turnover ratio, NE Treatment- $F(2,48)=7.97$, $P<0.01$, NE/MHPG Treatment- $F(2,48)=21.71$, $P<0.001$, with an effect seen in low dose subjects. A sex-treatment interaction was observed in decreased concentration of NE in low dose males, Sex x Treatment- $F(5,45)=5.36$, $P<0.01$; increased concentration of MHPG in low dose females, MHPG Sex x Treatment- $F(5,45)=15.29$, $P<0.001$, and increased turnover ratio of NE/MHPG in low dose males and females, Sex x Treatment- $F(5,45)=6.85$, $P<0.01$

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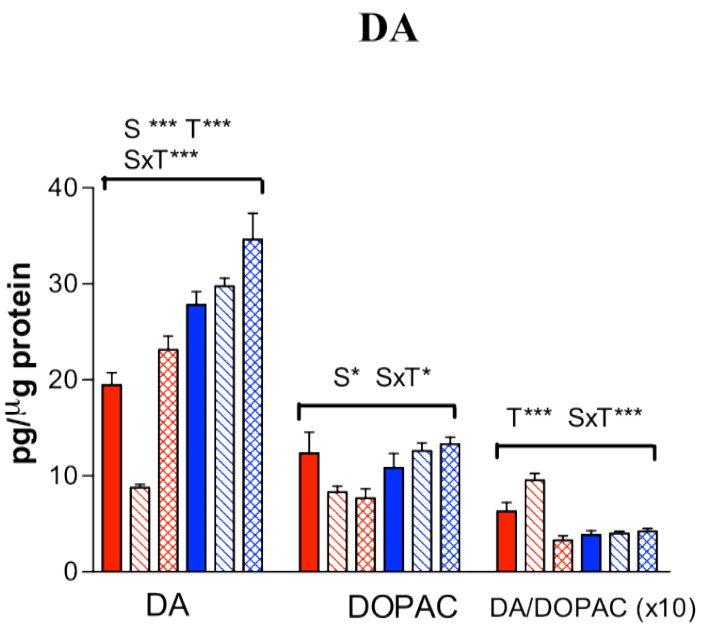
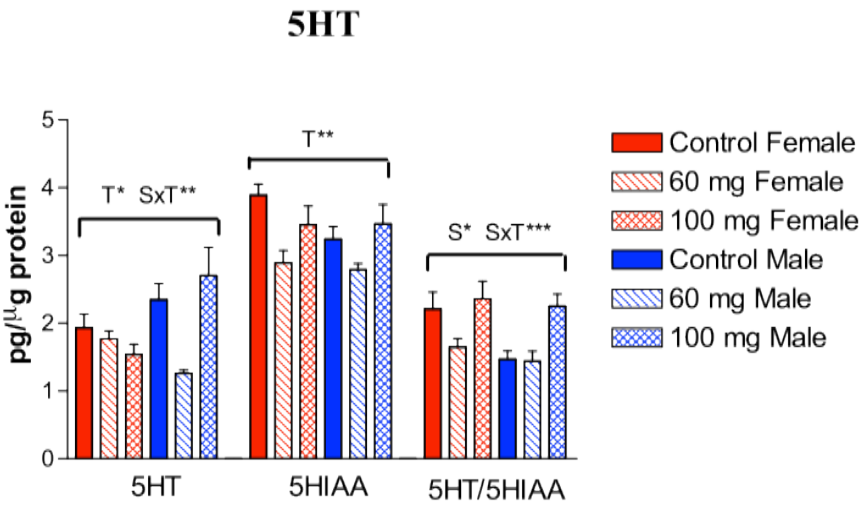
Figure 18 b



Concentration and turnover ratio of dopamine and its metabolite, DOPAC, in medial preoptic area. DA: A sex difference was observed in DA and DOPAC concentration, DA Sex- $F(1,49)=113.13$, $P<0.001$, DOPAC Sex- $F(1,49)=6.37$, $P<0.05$, with higher concentration in males. A treatment effect was observed in DA concentration, Treatment- $F(2,48)=18.85$, $P<0.001$, and DA/DOPAC turnover ratio, DA/DOPAC Treatment- $F(2,48)=14.41$, $P<0.001$, affecting low and high dose subjects. A sex-treatment interaction was observed in concentration of DA, Sex x Treatment- $F(5,45)=8.79$, $P<0.001$, DOPAC, Sex x Treatment- $F(5,45)=3.83$, $P<0.05$, and DA/DOPAC turnover ratio, Sex x Treatment- $F(5,45)=12.38$, $P<0.001$. Low dose females were significantly affected.

Figure 18 c



Concentration and turnover ratio of serotonin and its metabolite, 5HIAA, in medial preoptic area. 5HT: A treatment effect was observed in 5HT and 5HIAA concentration, 5HT Treatment- $F(2,48)=4.49$, $P<0.05$, 5HIAA Treatment- $F(2,48)=6.44$, $P<0.01$. This effect was observed in low dose subjects. Low dose subjects had decreased concentration of 5HT and 5HIAA. A sex-treatment interaction was observed in 5HT concentration, Sex x Treatment- $F(5,45)=6.33$, $P<0.01$ and 5HT/5HIAA turnover ratio, Sex x Treatment- $F(5,45)=8.68$, $P<0.001$. Low dose females had decreased 5HT/5HIAA turnover. Low dose males had decreased 5HT concentration, and increased 5HT turnover was observed in high dose males. A sex difference was observed in 5HT/5HIAA turnover ratio, 5HT/5HIAA Sex- $F(1,49)=4.80$, $P<0.05$, with higher activity measured in females.

Striatum (Figure 19 a,b,c)

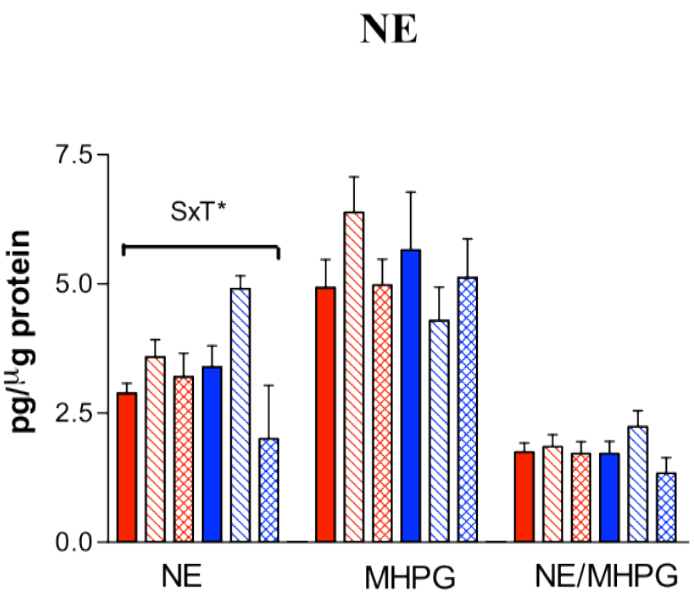
NE/MHPG: No main effect was observed in NE/MHPG concentrations or turnover. A sex-treatment interaction was observed in NE concentration. 60 µg males had significantly increased NE concentration.

DA/DOPAC: A treatment effect was observed in concentration of DA and DOPAC. 100 µg subjects DA concentration was significantly higher with respect to 60 µg subjects. DOPAC concentration in 60 µg subjects was significantly lower with respect to both 100µg and control subjects.

5HT/ 5HIAA: A sex difference was observed in 5HT. Males had increased concentration of 5HT with respect to females.

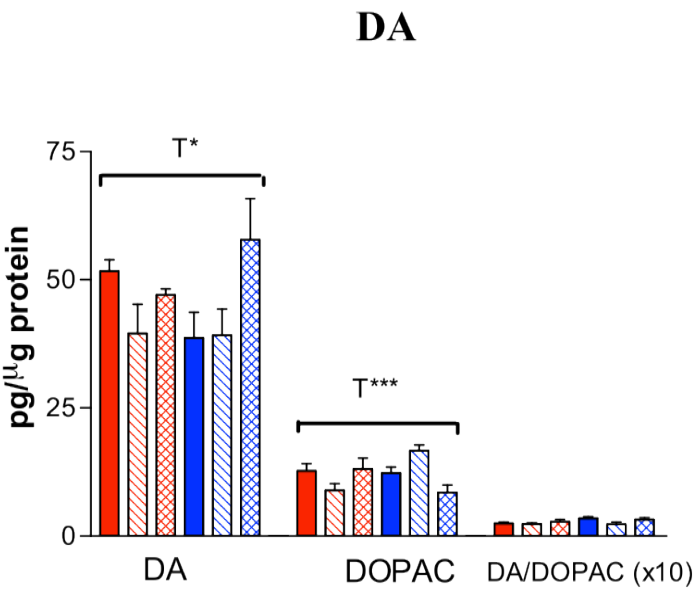
Figure 19 a

Striatum



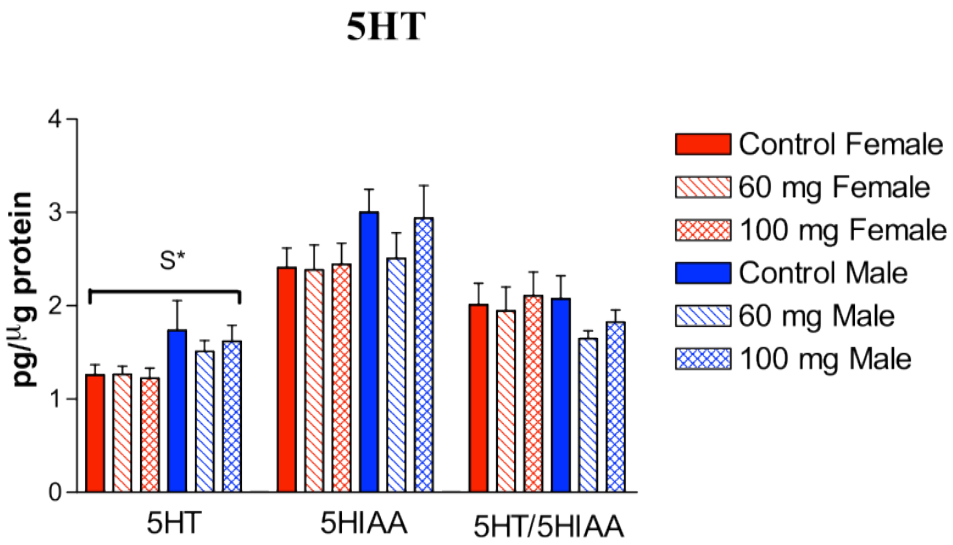
Concentration and turnover ratio of norepinephrine and its metabolite, MHPG, in striatum. NE: A sex-treatment interaction was observed in NE concentration, NE Sex x Treatment- $F(5,45)=5.02$, $P<0.05$. Concentration was increased in low dose males.

Figure 19 b



Concentration and turnover ratio of dopamine and its metabolite, DOPAC in striatum. DA: A treatment effect was observed in DA, DA Treatment- $F(2,48)=3.50$, $P<0.05$, and DOPAC concentration, DOPAC Treatment- $F(2,48)=8.36$, $P<0.001$. This effect was observed in low dose subjects.

Figure 19 c



Concentration and turnover ratio of serotonin and its metabolite, 5HIAA, in striatum.5HT: A sex difference was observed in 5HT concentration, 5HT Sex- $F(1,49)=5.56$, $P<0.05$, with greater concentration in males.

VIII. Discussion (Experiment 2- Postnatal BPA Exposure)

This experiment investigated the effects of BPA exposure at a later time period in development, from postnatal day 0 to 6, and at two doses, 60 μg and 100 μg/kg body weight. We hypothesized that the effects of postnatal BPA would be similar to those in the prenatal experiment. The data shows that postnatal exposure to BPA has significant physiological effects in female rats, but not in male rats. High dose BPA exposure (100 μg/kg) in the neonatal period was associated with increased body weight, and delayed date of vaginal opening. Previous studies support our finding of advanced age at time of vaginal opening, and increased body weight in adult females (George, et al, 2003; Rubin, et al, 2001). These findings further suggest that BPA exposure may interfere with weight set-point not only during the prenatal period, but also during the postnatal period, and that postnatal BPA exposure may disrupt hormonal signaling involved in puberty onset.

Neonatal exposure to BPA induces behavioral and cognitive changes in both sexes. These changes are organizational in nature, and therefore persist until adulthood. In our study,100 μg BPA treated females exhibited decreased anxiety in elevated plus maze performance, which was the opposite effect of what we hypothesized. A trend toward increased anxiety in high dose male subjects was observed, although this result was not statistically significant. In a study by Patisaul and Bateman, exposure to 50 micrograms BPA from postnatal day 0 to 4 increased anxiety in male rat pups (2008). Our finding, although not statistically significant, is in concert with existing data. As previously referenced, In untreated, intact animals, male rats have a

tendency toward higher anxiety in elevated plus maze when compared to female rats (Johnston and File, 1991). The mechanism by which BPA affects the anxiety centers in the brain appears to be sex specific. BPA exposure in prenatal critical period exaggerates the existing sex difference in anxiety behaviors of rats.

Subjects exposed to BPA prenatally had deficits in both spatial and visual memory. In our postnatal study, visual memory was not significantly affected by BPA exposure, and impairment in spatial memory task performance was observed only in 100 μg females. Poor spatial memory task performance suggests cognitive impairment in these females. Males showed no memory enhancement or impairment as a result of BPA exposure. This would suggest that males were protected against the effects of BPA seen in female subjects. It is possible that the postnatal testosterone surge may have conferred some neuroprotection in male subjects. Females were more affected by the exposure to BPA than males. The observed behavioral changes, as those observed in the anxiety measurements, appear to be an exaggeration of the sex-specific behavioral traits, as males tend to have better performance in spatial memory tasks, and exhibit increased anxiety behavior at this stage of life (Carr, et al, 2003; Doermus, et al, 2006). There are few existing studies on the cognitive effects of postnatal BPA exposure, specifically in learning and memory. In the study by Carr, administering 100 micrograms of BPA to female pups from postnatal day 1 to 14 abolished the sex difference in performance on the Morris Water Maze—these females improved their performance in the task. Higher doses of BPA (250 micrograms) had deficits in maze acquisition (2003). The results in the Carr study are dose-dependent, as were those in our study. However, we observed spatial memory deficits at the lower dosage, and with

a shorter period of exposure. It is possible that due to differences in drug administration (subcutaneous injection VS Carr, et al: Oral administration), our subjects had higher serum concentrations of BPA, and therefore, had more deleterious effects.

Neurochemical results support organizational neural changes as a result of neonatal exposure to BPA. Female subjects had changes in NE/MHPG turnover, DA concentration and turnover, and 5HT turnover in basolateral amygdala (BLA), a region involved in anxiety behavior. This suggests that neonatal exposure to BPA exerts organizational effects on this brain area, which was reflected in females' behavior. The amygdala, specifically its basolateral complex, is a crucial area of the brain for processing learned emotional response, such as anxiety and fear behaviors (LeDoux, 1992). In our study, the changes in monoamine levels and turnover were dose-dependent in this brain area. 100 microgram BPA treated females had a decrease in NE and 5-HT turnover, and increased DA turnover, while 60 microgram BPA treated females had the opposite effect, decreased DA turnover and increased NE and 5-HT turnover. These females had no changes in anxiety in elevated plus maze. Physiologically, NE has an important role in anxiety and pain response; and irregularities in the 5-HT system may contribute to anxiety disorders (Cooper, et al, 2003), so it is plausible that this result may contribute to the observed behavior.

100 μg BPA treated females had impairment in spatial memory tasks, and significant treatment effects were observed in CA1 and CA3, areas important in cognition. In CA1, we observed increased concentration of NE, and in CA3, and increase in NE turnover and DA concentration.

No significant changes were observed in striatum of 100 μg females, an area important in cognitive planning. In this instance, the neurochemicals measured do not appear to contribute to BPA's effect on spatial memory. Noradrenergic neurons and their projections have been found to contribute to learning and memory, especially in the area of global orientation (Cooper, et al, 2003). Few studies exist directly measuring the effects of BPA on NE, DA and 5-HT concentration and turnover in the brain. In a study by Honma, et al, increased turnover of DA and 5-HT was observed in pups treated with 4 or 40 mg/kg/day of BPA, however, many of the measurements were taken from mixed samples (forebrain, hindbrain, whole brain), and at non-environmentally relevant levels (2006).

Males and females showed increased activity in NE, DA, and 5HT systems in the MPOA of the hypothalamus, a sexually dimorphic area important in behaviors related to reproduction. This result suggests that this area is highly sensitive to neonatal BPA exposure in both sexes. The effects do not appear to be a clear feminization/masculinization effect as far as we can tell. No sex behavior studies were included in this investigation, however, in light of the effects seen in MPOA, this may be an important area for future study. The MPOA is critical in generating LH cyclicity (Gray, 1978), and thus the BPA-induced changes in monoamines may contribute to the delay in vaginal opening and puberty we observed. The changes in MPOA are important because they are consistent with the changes that others report in this area and in the hypothalamus.

Existing neurochemical data (Honma, et al) supports our hypothesis of neural changes as a result of BPA exposure. We administered BPA directly to pups in the critical period for sexual

differentiation of the brain, in environmentally relevant levels. BPA doses tested in published studies are well above recommended safety dose of 50 micrograms per day, and in some cases are toxic doses. In existing studies, pups were exposed to BPA for significantly longer periods of time. In several studies, especially those involving monoamine analysis, pups were not allowed to reach maturity, and therefore there is little published data establishing whether these changes persist to maturity. We believe our study is novel, in that BPA was administered in environmentally relevant levels, at specific critical periods in brain development; and persistent changes were found in mature animals, both in integrated behavior and monoamines in specific brain areas.

IX. General Discussion

Bisphenol A (BPA) is a compound used in plastics, coatings and resins, which, when ingested has been shown to affect several body systems and tissues, especially during development. We investigated whether exposure of rats to this compound during the prenatal and postnatal period of sexual differentiation of the brain would affect physiological measures such as body weight and puberty onset, anxiety behavior, locomotor activity, performance in cognitive tasks, and neurochemical profiles at adulthood.

In the first experiment, pregnant dams were administered wafers treated with 100 μg of BPA per kg of body weight, from gestational day 16 to parturition. 32 Male and female pups were weaned and at postnatal day 21, female pups were examined for evidence of vaginal opening. At two months of age, behavioral testing began with elevated plus maze (anxiety), open field (locomotor activity, hyperactivity, exploratory behavior), object recognition (visual memory) and object placement (spatial memory). Reproductive organs were then harvested and examined for morphological changes. Significantly low juvenile body weight was observed in BPA treated pups. At adulthood, BPA treated males were significantly overweight when compared to their control counterparts. Subjects exposed to BPA exhibited significant hyperlocomotion, and deficits in both visual and spatial memory tasks.

A second experiment investigated the effects of low and high doses of BPA exposure later in the critical period of sexual differentiation of the brain. On postnatal day 2, 4 and 6, 50 pups received subcutaneous injections of saline (control), 60 μg/kg BPA (low dose), or 100 μg/kg BPA

(high dose). Pups followed the same experimental protocols as the previous experiment. BPA delayed vaginal opening in high dose female subjects (Day 33.5) with respect to both low dose and control subjects. The adult body weight of high dose females was significantly higher than control counterparts. Males showed no significant weight difference. Significant increases in locomotor activity in open field task were observed in both male and female high dose BPA subjects. High doses of BPA had sex-specific effects on anxiety. High dose females showed significantly less anxiety in elevated plus maze performance. High dose females spent significantly more time in open arms of the maze, and had significantly greater number of open arm entries with respect to both low dose and control females. BPA treated males showed no significant differences in elevated plus maze performance as compared to control males. Exposure to high doses of BPA also induced changes in performance on spatial memory in female subjects. High dose females showed significant impairment in object placement spatial memory task with respect to control females. BPA treated males showed no significant differences as compared to control males in performance on spatial memory tasks.

Neurochemical analyses through HPLC showed increased activity in the norepinephrine system in CA3 of hippocampus (low dose females) and basolateral amygdala (high dose males and females), and increased activity of the dopaminergic system in basolateral amygdala (high dose males and low dose females). In addition, several changes including increased dopamine and norepinephrine system activity, and decreased 5HT were found in the MPOA area which is important in regulating the HPG axis.

These results suggest that prenatal BPA exposure is associated with hyperactivity, memory deficits and low juvenile weight in both sexes, and overweight in males. Postnatal BPA exposure is associated with behavioral, cognitive and physiological changes in female rats, and neurochemical changes in both sexes. Additionally, female rats experience greater effects from neonatal BPA as compared to males. This suggests that males experience increased sensitivity to BPA during an earlier developmental window. Results also suggest that exposure to BPA in prenatal and early neonatal life results in changes that persist to adulthood.

In this study, we collected data across physiological, behavioral and neurochemical parameters. BPA doses used in this study were more environmentally relevant (closer to EPA "safety" dose of 50 μg/kg/day, well below "no observed adverse effects" dose of 50 mg/kg/day) than many published studies investigating cognitive and neural effects. We had several significant findings: In our neonatal study, females, having no circulating estrogen at this stage of development, were more sensitive to BPA exposure than males. Males experience a surge of testosterone soon after parturition, which can be aromatized to estrogen in the brain (MacLusky & Naftolin, 1981). Anti-thyroid properties of BPA may have contributed to the outcome we observed in our study. BPA has been shown to have anti-thyroid effects (Zoeller, et al, 2005). At this developmental time point, thyroid hormone deficiency can lead to cognitive deficits (Zoeller & Rovet, 2004). However, the fact that a sex difference was seen in the effects of BPA exposure would lead one to reason that it is more likely an interaction with gonadal hormones. If the hypothesis that BPA is acting on the estrogen receptor to produce the deficits seen in females, the relative low affinity

of BPA for the estrogen receptor (Krishnan, et al, 1993), coupled with the high levels of testosterone, may have been responsible for the lack of significant behavioral changes in males.

In our prenatal study, males and females were equally susceptible to the effects of BPA exposure. In addition to estrogenic and thyroidogenic effects, BPA has been found to inhibit steroidogenesis (Akingbemi, et al, 2004) and affect the pituitary system in males (Nakamura, et al, 2010). Anti-androgenic effects of BPA may account for the cognitive and behavioral changes seen in male subjects. Previous studies have shown that hyperactivity (Masuo, et al, 2004) and fear behaviors (Negishi, et al, 2004) have been significantly affected in both sexes by developmental exposure to BPA, however these studies observed the effects of long-term BPA exposure in neonates, and the mechanism by which BPA induced these changes was not determined. It appears that males are less susceptible to the behavioral effects of BPA at specific developmental timepoints, which would lead us to a conclusion that BPA has interaction with the gonadal hormone system, rather than the thyroid system, to produce these changes. Further investigation into the mechanisms underlying these findings is necessary, as there is little existing evidence that shows cognitive impairments to be a result of BPA action on the estrogen or androgen receptors exclusively.

BPA is a ubiquitous compound, found in baby bottles, toys, plastic food containers, metal cans, dental fillings and even ground water. It is important to look at the larger implications of exposure to BPA. The incidence of autism spectrum disorders (ASD) and ADHD has increased greatly in the past few decades. Although there is no specific pathology that has been found to

cause these conditions, many brain areas and neurotransmitters are believed to be involved in aspects of these conditions.

Problems with DA regulation in frontal cortex have been observed in children with ADHD (Takeuchi, 2008), specifically, an imbalance in the noradrenergic neurons regulating dopamine function in frontal cortex. In autism, DA, 5HT and glutamate disregulation in several additional brain areas, including the amygdala, striatum, in addition to frontal cortex, are believed to play a role in the etiology of the disorder (Baron-Cohen, et al, 2000). Zhou, et al (2009), found that perinatal BPA exposure resulted in long-term potentiation and depression deficits in the striatum of rats, and alterations in DA receptors induced by BPA exposure. Neurochemically, in our study, both males and females experienced organizational changes as a result of neonatal BPA exposure. In the postnatal BPA study, we observed significantly higher concentrations of NE in the frontal cortex of BPA treated subjects (males), as well as increased DA in basolateral amygdala (males and females) and striatum (males). These findings do agree with established BPA studies and ASD studies of brain monoamines. Significant behavioral deficits were not observed in the males in this study. Females, however, exhibited significant cognitive deficits in both studies, and males exposed to BPA at our earlier developmental window did exhibit hyperactive behavior and cognitive deficits, although no monoamine data is available for these subjects.

In a study by Simon Baron-Cohen, he empirically investigated the theory of autism as an "extreme male brain" (EMB) syndrome, a concept originated by Hans Asperger in the 1940s. He

found that many autistic male individuals experience precocious puberty and have elevated serotonin levels, both of which can be linked to elevated fetal testosterone (Knickmeyer R, Baron-Cohen S, 2005). During the critical period of sexual differentiation of the brain, fetal testosterone is aromatized to estrogen in the brain, which triggers masculinization (McEwen, et al, 1977). It is possible that exposure to an estrogen mimic, during this same period of development, may also activate the estrogen receptors that trigger this process. If the effect of BPA was additive, it seems plausible that the brain could become hyper-masculinized. We observed increased 5HT activity in MPOA, a sexually dimorphic brain region involved in LH signaling, in BPA treated males in the postnatal study. Males' behavior was most significantly affected in the prenatal study, however we have no neurochemical data for these subjects. The masculinized brain theory of ASD has been applied also to females with these disorders (Ingudomnukul, et al, 2007). Masculinization may have played a role in the physiological changes in our female BPA treated subjects. Females in our postnatal study had significant changes in DA, NE and 5HT systems in MPOA.

Alterations in monoamines in MPOA may have contributed to the delay in vaginal opening and overweight observed in this study. Delayed onset of menstruation and elevated serum testosterone has been observed in females with ASD (Knickmeyer, et al, 2006; Tordjman, et al, 1997). Positive feedback in the NE and DA systems in hypothalamus 20 days after parturition in rats initiates the first LH surge, bringing about puberty (Wuttke, et al, 1980). If BPA exerts an organizational change in the NE and DA systems in MPOA, this may interfere with LH cycling, and pubertal onset. BPA increases glucose uptake (Sakurai, et al, 2004), and blocks the release

of adiponectin, an adipocyte hormone that increases sensitivity to insulin and inhibits inflammation (Hugo, et al, 2008). The combination of these factors may contribute to metabolic syndrome, a condition characterized by increased body weight, insulin insensitivity, increased serum triglycerides, and heightened cardiovascular disease and diabetes risk (NHLBI). In a study by Lang, et al (2008), it was found that urinary levels of BPA directly correlated with coronary artery disease (CAD) and diabetes. The Barker Hypothesis, also known as "Thrifty Phenomenon" states that retarded fetal growth may lead to organizational changes that lead to increased risk of CAD, obesity, and diabetes (Barker, 1997). The observation of decreased juvenile body weight, followed by significantly increased adult weight in the male prenatal BPA subjects is in agreement with this hypothesis, and with the suspected mechanism of BPA action on adipose tissue. Exposure to PTU, a compound that blocks the production of thyroid hormone, leads to decreased growth, which resolves itself by puberty. No delay in date of vaginal opening or first estrous was observed in these subjects (Wilen, Bastomsky and Naftolin, 1981). Females exposed prenatally to BPA did not have a delay in age at vaginal opening, and were not over or underweight when compared to their control counterparts, a finding which is in agreement with a possible anti-thyroid action of BPA.

The aim of this study was to investigate the physiological, behavioral, and neurochemical effects of exposure to endocrine disruptor Bisphenol A. Much further investigation needs to be done, in the areas of BPA exposure effects and in autism, ADHD, and obesity. It is highly unlikely that BPA is the sole cause or contributor to the ASD, ADHD and overweight, which is so prevalent in children and adolescents, or that only one mechanism of action is involved. Many factors,

genetic and environmental, may contribute. Also, BPA exerts effects in humans that include, but are not limited to, thyroid dysfunction (Heimeier, et al, 2009), infertility (Hauser R & Sokol R, 2008), and carcinogenic effects (Park, et al, 2009). Further studies must be conducted to determine the mechanisms by which BPA brings about these effects, and revisit the issue of "safe" BPA exposure.

X. **References**

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