THE IMMUNE AND NEUROLOGICAL IMPACTS OF DEVELOPMENTAL BISPHENOL A EXPOSURE IN A C57BL/6 MOUSE MODEL

By

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Environmental exposure to exogenous agents during critical time points of development may be associated with the onset of deleterious effects, including immune and neurological disorders. Numerous studies have shown that exposure to bisphenol A (BPA) can disrupt myriad biological systems. This research was focused on the immune and neurological impacts of developmental exposure to BPA both with and without an acute exposure to lipopolysaccharide (LPS) in adulthood. LPS exposure given around the time of learning "unmasks" developmental deficits in learning and memory induced by exposure to an exogenous agent earlier in life. We hypothesized that adult exposure to LPS would unmask BPA-induced developmental impacts to hippocampal-dependent learning and memory of C57BL/6 offspring. In one set of experiments, C57BL/6 female mice were exposed to 0, 25, 50, or 100 mg/kg of BPA in a corn oil vehicle by gavage, beginning at pairing with males and ending at weaning of offspring. The F1 generation were assessed on a Barnes maze at postnatal day 21 (PND21), 42, and 60. Splenic lymphocyte immunophenotype was evaluated after behavioral testing.

The behavioral results from this study included high intra- and intertrial variability across ages and between sexes. While this is not unusual for this type of behavioral measure, significant differences between ages and dose groups did not conform to any observable patterns

during acquisition learning. On the final reference day, exposure to BPA was associated with more correct attempts in the Barnes maze. The fewest number of correct attempts was observed in BPA-exposed females at PND60. Several significant differences in behavior were noted among age groups, and indicate that as these C57BL/6 mice aged, they responded differently to the same tasks. BPA exposure led to modified immune cell numbers at different doses and ages. Overall, this study demonstrated that BPA could alter behavior, but no consistent patterns emerged with regard to the effects of BPA dose or age at testing.

In another set of experiments, pregnant C57BL/6 female mice were exposed to 0, 0.4, or 50 mg/kg of BPA in a corn oil vehicle. The resulting offspring were assessed on a Barnes maze at PND60, beginning 4 hours after a single challenge with LPS. Mice developmentally exposed to different doses of BPA made more correct escape attempts than vehicle control animals; no control groups outperformed BPA exposed groups on any behavioral measure. Control animals injected with saline significantly outperformed LPS-challenged animals. Female animals were 88% faster at this visuospatial task than males. This is inconsistent with previously reported studies where males outperform or perform equally with females on this type of evaluation. BPA exposure could be associated with impaired spatial memory in males on this task. We also reported significant changes to NK cell numbers, CD4+CD25+ T cell numbers in females and alterations to IgG in males and IL-4 in female mice. Overall, our findings suggest that developmental BPA exposure, can alter learning and immune cell types but additional research should be conducted to determine at which doses these alterations occur.

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A Dissertation

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Pharmacology and Toxicology

By

Jason Neil Franklin

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DEDICATION

This work is dedicated to my family; especially to my wife, Sheri and daughter, Lillian.

I love you both very much.

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I would like to thank God for everything he has done for me during the pursuit of my PhD at East Carolina University. Through Him all things are possible and I would be amiss if I did not acknowledge His gifts first and foremost when considering those that have helped me to get to where I am today.

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LIST OF SYMBOLS OR ABBREVIATIONS

APC: Allophycocyanin

BALB/c: Bagg Albino inbred mice

BPA: Bisphenol A

C57BL/6: C57 black 6 inbred mice

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CD25: cluster of differentiation 25

CNS: central nervous system

DES: diethylstilbestrol

ELISA: enzyme-linked immunosorbent assay

ER: estrogen receptor

FACS: fluorescence-activated cell sorting buffer

FITC: Fluorescein isothiocyantate

HEL: Hen egg lysozyme

HRP: horse radish peroxidase

IgG: Immunoglobulin G

IFN-γ: interferon-gamma

IL-4: interleukin-4

IL-10: interleukin-10

IL-1β: interleukin-1 beta

LPS: lipopolysaccharide

m: minute

mAb: monoclonal antibodies

mg/kg: milligram per kilogram

NK: Natural Killer Cells

OVA: ovalbumin

OVA-TCR-Tg: Ovalbumin-specific T-cell receptor trans-genic mouse

PE: phycoerythrin

PND21: postnatal day 21

PND42: postnatal day 42

PND60: postnatal day 60

s: second

TNFα: tumor necrosis factor alpha

CHAPTER ONE – GENERAL INTRODUCTION

1.1. Etiology of developmental disorders

Neurodevelopmental disorders refer to numerous diseases or diagnoses associated with behavioral disturbances that are present from birth or with symptoms that develop at an early age. These disorders are characterized by a combination of symptoms including deficits in learning, communication challenges, mental and sometimes physical retardation or impairment. Even though developmental disorders or their related symptoms have been reported since the beginning of written history, the etiology for these disorders is not clearly understood.

One of the developmental disorder diagnoses is autism spectrum disorder (ASD). This disorder is associated with complex neurodevelopmental symptoms including diminished social interaction, deficits in verbal and non-verbal communication and repetitive or restricted interests or patterns of behavior (Ashwood et al., 2008). While current research suggests a genetic link for some of those that develop this disorder, other research indicates that those with ASD have higher incidence rates of immunological alterations compared to the general public (Lyall et al., 2014). It is unclear what causes these immune changes, though a few genetic markers are linked to both these immune alterations and other symptoms associated with the diagnosis. While these abnormal immune responses exist in those with ASD it is unclear whether these immune changes are the result of the diagnosis or if they play a role in the cause of behavioral symptoms.

Some theorize that alterations to the immune response could lead to inflammation and autoimmunity, which could be the potential basis for some cases of ASD and potentially other types of neurodevelopmental disorders (Ashwood et al., 2008). If the etiology of ASD or other

neurodevelopmental disorders is linked to immune dysfunction, it would be possible for exposure to environmental xenobiotics to play a role in inducing or exacerbating immunotoxicity and leading to downstream neurotoxicity (Ashwood and Van de Water, 2004). The most likely xenobiotic to play a role in immune-induced neurotoxicity would be a toxicant or combination of agents that targeted receptors or signals important to both developing immune and central nervous system (CNS) cells. This agent would be able to cross the placental barrier and alter developing immune and nervous system cells, receptors, or signals, causing lifelong alterations to the structure and plasticity of cells in the CNS (Ashwood and Van de Water, 2004).

The toxicant bisphenol A (BPA) is known to target both immune cells and CNS cells by binding to estrogen receptors on these cell types (Brinton, 2009; Pierdominici et al., 2010; Huang et al., 2014). This chemical readily crosses the placental barrier and is present in cord blood samples and in amniotic fluid (Ikezuki et al., 2002; Domoradzki et al., 2004; Richter et al., 2007; Nishikawa et al., 2010). These are the primary reasons why we chose to evaluate the effects of developmental BPA exposure and why we are interested in both learning alterations and immune changes in the same experimental model.

1.2. History of bisphenol A

BPA (4, 4'-isopropylidene-2-diphenol) was originally synthesized in 1891, by Russian chemist Aleksandr P. Dianin, who combined phenol with acetone (Figure 1.1) in the presence of an acid catalyst to produce the chemical (Rogers, 2014). BPA was then recreated in the 1930s and was designed as one of several compounds with estrogenic activity but was overshadowed by diethylstilbestrol, which showed stronger estrogenic activity (Dodds and Lawson, 1936;

Alonso-Magdalena et al., 2012). In the 1950s BPA was found to react with phosgene (carbonyl chloride) to produced a clear hard resin known as polycarbonate plastic (Rogers, 2014).

Polycarbonate plastics have risen in popularity through the years for use in a wide variety of products due to the fact that they are very durable and heat resistant. Polycarbonates are used in microwavable dishes, and in a variety of safety equipment like sports visors and automotive equipment due to its resistance to high impact collisions (Rogers, 2014). BPA is also used in the food industry in the form of epoxy resins used to seal and coat the interior of canned goods in an effort to increase the shelf life of goods (Rogers, 2014). Products like eyeglasses and windshields use BPA because of its clarity and durability and it is used in composite papers including some forms of currency due to its thermal and water resistance (Liao and Kannan, 2011; Hormann et al., 2014). Widespread popularity and diverse utility has lead to BPA becoming one of the highest volume chemicals produced worldwide (Alonso-Magdalena et al., 2012). Approximately five million metric tons of BPA are produced annually to create these consumer products and as a result, this chemical has become a widespread pollutant that can be detected in soil, water and dust samples around the world (Burridge, 2008; Loganathan and Kannan, 2011; Liao et al., 2012; Michalowicz, 2014).

BPA is regarded by the U.S. Environmental Protection Agency (US EPA) as a reproductive, developmental, and systemic toxicant in animal studies (Bisphenol A, 2011). Due to reproductive and developmental risks, the Food and Drug Administration has banned the use of BPA in products intended for children under the age of three, including bottles and sippy cups after similar policy bans have limited this chemical's use in a dozen states, in China, and in Canada (Koch, 2012).

1.3. Routes of exposure and metabolism

The primary source of human BPA contact is believed to occur through ingestion of contaminated food and beverages from polycarbonate containers or epoxy resins that leached BPA into food stuffs (Kang et al., 2006b; Zalko et al., 2011; Donohue et al., 2013). Leaching of BPA occurs with reuse or wear of polycarbonates. Heat and acidic or basic conditions used to clean and sanitize these containers can accelerate hydrolysis of the ester bond in BPA monomers, increasing the rate of BPA leaching into the food or beverages contained in BPA constructed containers (Yu et al., 2011). Secondary dermal absorption is also possible as BPA is a lipophilic compound and can be absorbed through the skin (Liao and Kannan, 2011; Michalowicz, 2014). BPA is present in thermal paper used for tickets and cash register receipts. Dermal absorption is increased after use of hand sanitizer, as may occur between contact with thermal paper source in a restaurant and sanitizing hands prior to eating (Hormann et al., 2014).

BPA is readily metabolized in mammals and is rapidly cleared through first past hepatic metabolism. Free BPA has a reported half life of 5.3 hours and can be predominantly cleared from the human body in 24 hours (Volkel et al., 2002). BPA is primarily conjugated to create BPA glucuronide that is no longer an estrogen receptor binding compound. BPA glucuronide is primarily excreted in urine in humans and in feces in rats and mice (Figure 1.2). BPA glucuronide can be broken back down into the active free BPA by beta glucuronidase, but BPA is predominantly cleared before this takes place (Volkel et al., 2002). During development the placenta has a high rate of glucuronidation to clear BPA from circulation, but BPA that does pass the placental barrier is not as rapidly metabolized by fetal metabolism as glucuronidation is limited early in development. The predominant clearance in the fetus is through arylsulfatase C

to convert free BPA to BPA sulfate, which is a secondary metabolic pathway in adults (Kang et al., 2006a). BPA sulfate is also considered an inactive BPA metabolite, as it is unable to bind to estrogen receptors. Some BPA sulfate is converted back to free BPA by sulfo transferases, but this cycle greatly reduces free BPA exposure in utero (Matsumoto et al., 2002).

BPA is metabolized to many other "non-active" metabolites as well, including BPA diglucuronide, 5-hydroxy BPA, and 5-hydroxy BPA sulfate (Kang et al., 2006a). The number of different conjugates has made it challenging to compare the serum or urine BPA levels to determine the rate of BPA exposure. According to Vandenberg et al. (2010), a review of biomonitoring studies reported a median range of 0.3-4 ng/ml of unconjugated BPA in serum from men and women, but in order for Taylor and colleagues (2011) to reach these serum levels in experimental rhesus monkeys, they had to administer a dose by oral gavage of 400 µg/kg, or 0.4 mg/kg, which is much higher than the estimated 1 µg/kg per day for human exposures. This either means that humans are exposed to much higher concentrations of BPA per day than originally estimated, or additional routes of exposure to BPA that would avoid rapid first pass metabolism were overlooked (Taylor et al., 2011).

1.4. Study Aims

Previous studies on BPA exposure either during development or in adulthood demonstrated altered learning or memory performance (Goncalves et al., 2010; Jašarevic et al., 2011; Xu et al., 2013; Kumar and Thakur, 2014; Wang et al., 2014). Additionally, BPA was associated with alterations to the immune system (Ohshima et al., 2007; Yan et al., 2008). However, no studies have evaluated the effects of developmental BPA exposure subsequent outcomes in both the

nervous and immune systems simultaneously. Changes in both systems would indicate the possibility for neuro-immune signaling that could be associated with altered brain development. The present experiments tested the central hypothesis that **developmental exposure to BPA would be associated with changes to hippocampal-dependent performance on a test of spatial learning and that those changes would be associated with developmental immunotoxicity**.

Chapter two addresses the first aim of my research to **determine the effects of BPA on neurological development by the evaluation of hippocampal-based learning and memory and impacts on the immune system by assessing the immunophenotype of adaptive and innate cells associated with maturation of the immune and nervous systems.** This chapter focuses on animals with resting immune systems and addresses these specific questions:

1. Will developmental BPA exposure alter C57BL/6 mice learning rate or behavior during a visuospatial learning assessment?
2. Will developmental BPA exposure alter reference day behavior after the task has been learned?
3. Does developmental BPA exposure produce dose-dependent effects on immunophenotype?
4. What implications do these results have for understanding neurodevelopmental toxicity or immunotoxicity of BPA?

Chapters three and four address research conducted to reassess learning and memory from the first aim. Results from behavior testing are discussed in chapter three and changes to immunophenotype are analyzed in chapter four. Animals in these studies received a lower dose of BPA (400 µg/kg) that more closely fits with human exposure levels according to Taylor et al.

(2011). Chapter three evaluates BPA exposure during prenatal and postnatal periods and alterations to hippocampal dependent performance on a test of spatial learning and memory once the animals reached adulthood, PND60. Animals were assessed in a "double hit" model where they were developmentally exposed to BPA and then given an acute exposure to lipopolysaccharide (LPS) around the time of learning. LPS triggers an inflammatory response via induction of the cytokine interleukin-1β that can interfere with memory formation (Bilbo et al., 2005). We hypothesized that exposure to LPS would unmask BPA-induced developmental impacts to hippocampal-dependent learning and memory. This chapter addressed the following questions:

5. Will developmental BPA exposure alter C57BL/6 mice learning rate or behavior during on a visuospatial learning assessment?
6. Will the use of a "double hit" model using LPS uncover different BPA dose effects?
7. What implications do these results have for understanding neurodevelopmental toxicity with regard to developmental BPA exposure?

Chapter four addresses the other half of this study and reports on whether BPA exposure during prenatal and postnatal periods would alter the innate or adaptive immune cell response to a peripherally injected LPS challenge in adulthood.

The use of 0 mg/kg and 50 mg/kg dose groups across the initial pilot study and subsequent experiments provided replicate data for comparing behavioral outcomes among these studies. Statistical analysis of these replicates showed that within age, within sex, and within dose, behavioral endpoints did not significantly vary from study to study. This replicate consistency carried across all three studies.

Not all of my proposed aims were addressed by my research studies. Plans to measure changes to hippocampal morphology were not completed after my attempts to optimize a Golgi staining procedure failed. Brains for this assessment were removed at the conclusion of behavior testing and fixed in paraformaldehyde. Initially, no staining was apparent after incubation with silver nitrate. Adjustments were made to the protocol to incrementally increase staining time, but even with a 3-fold increase in incubation time, the morphology of these cells, particularly dendritic length and arborization, was not clear enough to measure for comparisons.

Figure 1.1. The chemical structure of Bisphenol A.

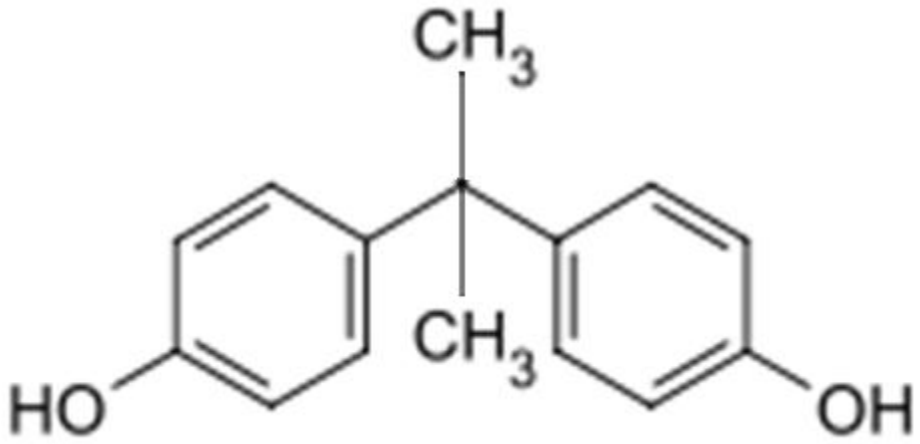
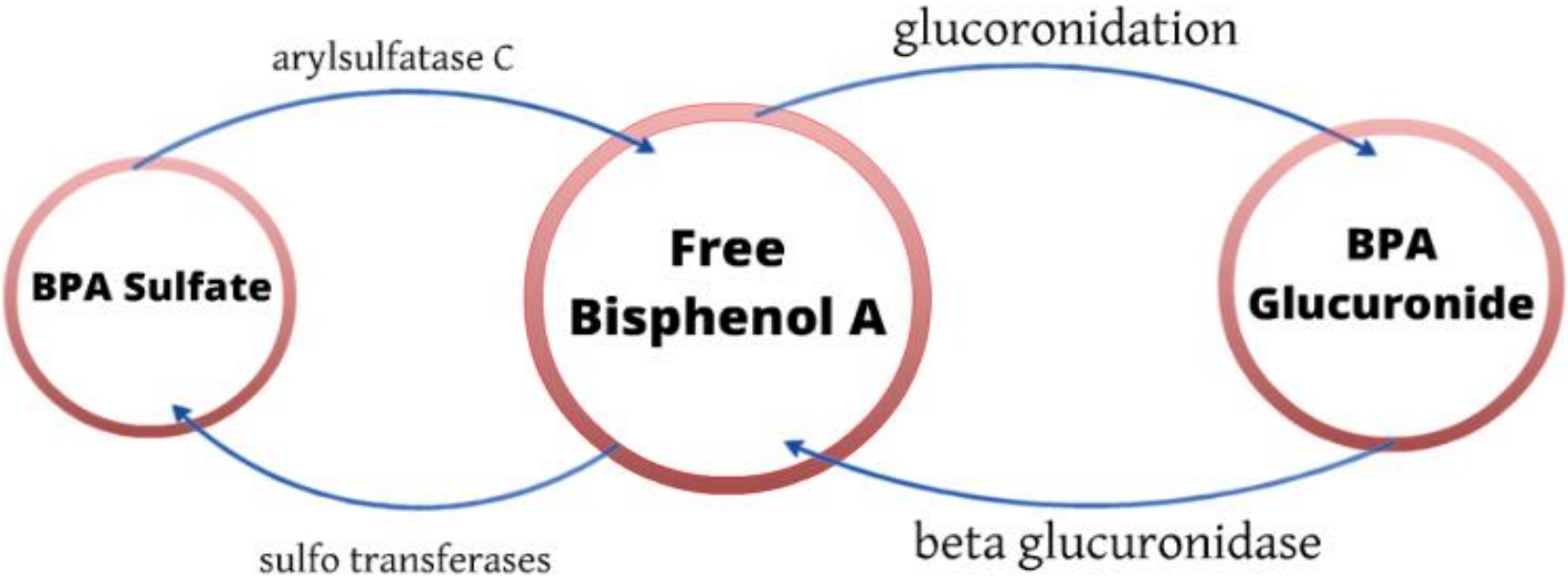

CH3
CH3
HO
OH

Figure 1.2. Primary metabolic pathways for BPA.


arylsulfatase C
glucoronidation
BPA Sulfate
Free
Bisphenol A
BPA
Glucuronide
sulfo transferases
beta glucuronidase

CHAPTER TWO – DEVELOPMENTAL BISPHENOL A EXPOSURE IN A C57BL/6 MOUSE MODEL INDUCES ALTERATIONS IN SPATIAL MEMORY AND IMMUNOPHENOTYPE

2.1. Introduction

Thousands of children born each year will ultimately be diagnosed with a developmental disorder that will drastically limit their capacity to learn, communicate, and function. Despite decades of research, the etiology of these disorders is widely unknown. Exposure to exogenous agents during critical windows of development may impact brain development and alter the ways that learning occurs. Early exposures may disrupt cell maturation, migration, differentiation, or function, resulting in neural damage or lifelong changes in neural signaling.

Estrogen and androgen signaling regulates the development and function of numerous physiological systems, including the central nervous system and the immune system (Weinstein et al., 1984; Kubo et al., 2001; Nalbandian and Kovats, 2005; Brinton, 2009; McEwen et al., 2012). Estrogen receptors have been identified on numerous cell types in both systems, including microglia, astrocytes, and neurons in the CNS and on lymphocytes including T cells, natural killer (NK) cells, and B cells (Brinton, 2009; Pierdominici et al., 2010; Huang et al., 2014). Agents that interfere with endogenous signaling to these receptors and other receptors in the endocrine system are commonly referred to as endocrine disruptors. By interfering with endogenous signaling to these cells endocrine disruptors could induce changes that result in long-term system limitations (Goto et al., 2007; Guo et al., 2010; Roy et al., 2012). Bisphenol A (BPA; 4, 4'-isopropylidene-2-diphenol) is one of the most widely studied endocrine disrupting

chemicals and has been shown to bind to estrogen receptors (ER) and disrupt endogenous hormone signaling (Kuiper et al., 1998; Wetherill et al., 2007; Yu et al., 2011). More recently new data has indicated a correlation between BPA exposure pathways independent of ER binding, including epigenetic modulation of gene expression (Bromer et al., 2010; Yeo et al., 2013). More research about these pathways is needed, but these initial reports indicate that the mechanisms for BPA associated changes have not clearly been established.

BPA was initially used to mimic estrogen in a laboratory setting (Dodds and Lawson, 1936). Now, due to its chemical structure, BPA is primarily used in the mass production of clear and shatter resistant plastics used in computers, automobiles, food packaging and in epoxy resins for lining metal food cans. Approximately five million metric tons of BPA are produced annually to create these products and as a result, this chemical has become a widespread pollutant that has been found in water and dust samples (Burridge, 2008; Loganathan and Kannan, 2011; Michalowicz, 2014). The primary source of human BPA contact is believed to occur through ingestion of contaminated food and beverages (Kang et al., 2006b; Zalko et al., 2011; Donohue et al., 2013). Heat and acidic or basic conditions used to clean and sanitize these containers can accelerate hydrolysis of the ester bond in BPA monomers, causing leaching of BPA into the food or beverages contained in the plastics (Yu et al., 2011).

Changes in learning or memory performance are associated with BPA exposure (Goncalves et al., 2010; Jašarevic et al., 2011; Xu et al., 2013; Kumar and Thakur, 2014; Wang et al., 2014). Exposure to BPA can alter working memory on a variety of memory tasks including a Y maze task and the Morris water maze (MWM) and alter expected sex-related differences in performance (Carr et al., 2003; Tian et al., 2010; Kumar and Thakur, 2014). Jašarević and colleagues (2011) reported that prenatal exposure to BPA was associated with a significant

decline in spatial memory in male offspring when compared to controls. In this study, male and female deer mice were evaluated with a modified Barnes maze at PND60 and male offspring from control dams performed significantly better than control female offspring and male and female offspring exposed to BPA during development. This indicates that BPA exposure modified the cognitive performance of these mice to match the female mice in this study.

Several studies reported links between BPA exposure and alterations of cell morphology in regions associated with learning and memory. The CA1 region of the hippocampus is associated with learning tasks that test visual spatial memory. BPA administration leads to a significant decrease in spine synapses in the CA1 region of the hippocampus (MacLusky et al., 2005; Kunz et al., 2011; Eilam-Stock et al., 2012). Nakamura (2006) studied developing brain tissue by injecting BPA and bromodeoxyuridine (BrdU) into pregnant mice and compared BrdU labeled cells after mice were born. BPA injections were associated with shifts in the morphology of the neocortex where BrdU fluorescently labels new cell growth, were shown to increase in some areas of the brain and decrease in others compared to control animals (Nakamura et al., 2006). These studies indicate that BPA exposure was associated with changes in learning formation and to neuronal cells in the regions of the brain associated with visuospatial learning.

Alterations to the immune system are also of interest because studies show that the immune system plays an integral role in learning and memory (Ziv et al., 2006). Mouse models that lead to a depletion of T and B cell populations through irradiation or due to specific knockouts have been associated with poorer performance on the MWM and Barnes maze. Kipnis et al. (2004) and later Brynskikh el al. (2008) demonstrated that performance on the MWM improved to match controls only after isolated T cell populations were restored in these models. Developmental exposure to BPA can lead to significant differences in T cell populations

(Yoshino et al., 2004; Ohshima et al., 2007; Yan et al., 2008) but these previous studies designed to address BPA related memory changes did not evaluate immune cell differences in their models.

We hypothesized that developmental exposure to BPA would be associated with changes to hippocampal dependent performance on a test of spatial learning and that those changes would be associated with developmental immunotoxicity.

2.2. Materials and Methods

2.2.1 Animals

Adult male (24-26 days old) and nulliparous female C57BL/6 mice (24-28 days old) were purchased from Charles River Laboratories (Raleigh, NC) and delivered to the East Carolina University (ECU) Brody School of Medicine (BSOM) animal facility (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). Initially, males were housed singly and females were housed four to a cage and were allowed a 26 day acclimation period. After acclimation, males and females were paired into breeder groups with one male and two females per cage. Mice were housed in polycarbonate cages with corn cob bedding, soft bedding material, a hiding tube, and a plastic climbing ring. All mice were maintained in a 23° ± 3ºC environment with 30-70% relative humidity, and given *ad libitum* access to both food (5P00 Prolab RMH 3000) and water. Animals were maintained in a 12 h light/dark cycle (light, 0730-1930 hours; dark 1930-0730 hours). All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at ECU.

2.2.2 Dosing solution

Dosing solutions were prepared fresh once per week. The appropriate mg/kg concentrations of BPA (Sigma Aldrich, St. Louis, MO, USA) were administered as 0.1 ml of dosing solution per 10 g of body weight. Stock solutions contained 2.5, 5, or 10 mg/ml of BPA that had been sonicated (Branson, 2510) until dissolved into corn oil (Sigma Aldrich, St. Louis, MO, USA).

2.2.3 Treatment

Immediately prior to pairing, all mice were weighed and randomly distributed into dose groups so that no individual group statistically differed by body weight. Starting on the date of pairing with a breeder male, dams received either vehicle control (corn oil), 25 mg/kg, 50 mg/kg or 100 mg/kg per day of BPA. One group of dams was also used as a negative control and was weighed daily, but was not gavaged. Dams were weighed daily and given 0.1 ml of BPA or vehicle control per 10 g of body weight per day via gavage until the weaning of pups on PND21, except for the negative control group. Females $\geq$ 24 g in body weight were considered pregnant and housed separately from males and nonpregnant females. Males were removed and euthanized when the second female in the cage reached $\geq$ 24 g in body weight. Female and male mice that failed to reproduce were removed from the study and euthanized.

2.2.4 Offspring

On PND1, litters were weighed, sexed and culled to three males and three females per dam. When possible, litters with an insufficient number of male or female pups were backfilled with extra pups of the same age from dams within the same dose group. Offspring were weighed once per week until weaning at PND21. Weaned offspring were housed in same sex sibling groups when possible (up to three animals per cage) and each cage was assigned a random number identifier to blind researchers to dose and dam number. Offspring were given ear punches for identification purposes. When possible, one male and one female from each litter were used for

behavior and immune testing starting on PND21, then another pair on PND42, and again on PND60.

2.2.5 Long-term spatial memory

The Barnes maze (Noldus Information Technology, Leesburg, VA, USA) is a 122 cm diameter circular platform raised on a central support to 144 cm from the floor to deter animals from leaping to the ground in efforts to escape. Forty 4.9 cm holes are bored through the platform along the edge and are equally spaced with each other and the outer edge. A black plastic escape box (21.4 cm x 5.4 cm x 8.5 cm) is positioned under one of the 40 holes in the platform and in the same spatial location for the duration of experiments. The surface of the maze is white and brightly lit by overhead lights, which serve as a negative reinforcer to motivate mice to find and enter the dark escape box. The hole above where the escape box is placed is designated as the "escape hole".

The escape box was removed from the table and repositioned so that it was always in the same location in reference to the visual cues in the room. Four large shapes were cut from construction paper and adhered to walls for the duration of experiments. Other objects were carefully noted to make sure they were always in the same place in the room (brooms, trash can, curtain, and chair). A camera, door, and broom rack were also prominently visible in the room and served as visual cues that the mice could use to orient themselves when the start box was lifted. The platform and escape box were cleaned after every trial and the table was rotated 90 degrees after every cage was tested.

During the acclimation period that began on PND21, PND42, or PND60, all animals were placed in the center of the table, under an opaque cardboard start box for 10 seconds (s). When the boxed was raised, mice were guided to the correct hole and placed in the escape box for two minutes (m). Acquisition began two days after acclimation, which is the learning trial period of the Barnes maze. Mice were placed under the start box in the center of the platform for 10 s, the box was raised, and a mouse was allowed to explore the platform for three m (180 s). After three m, or if a mouse entered the escape box, the trial ended and they spent one m in the escape box before being returned to their home cage. If a mouse failed to enter the escape box within 180 s, the observer guided the animal to the escape hole and made sure the animal entered the escape box. During the trial, the amount of time to initially find the escape hole (latency) and the amount of time until a mouse entered the escape box was measured. The number of primary errors before locating the escape hole and the number of errors after finding the escape hole also were recorded. Acquisition consisted of four trials on each of four days, with at least 15 m rest between each trial for each mouse.

On the final day of testing a reference test was conducted. The escape box was removed from the platform and mice were given a 90 s trial. The number of times a mouse poked its nose into the escape hole, where the escape box had been, were recorded. Primary errors, total errors, and latency were also recorded.

2.2.6 Locomotor activity

Overall locomotion was assessed in a clear, 43.18 cm square polypropylene open field chamber where movement was assessed with autotracking software, Autotrack ATM3 4.65

(Columbus Instruments, Columbus, OH, USA). Approximately 15 m after the final test on the Barnes maze, mice were placed in the center of one of four identical open field chambers. Each mouse was allowed 180 s to explore the open area and the distance traveled was recorded.

2.2.7 Flow Cytometry

Spleens from developmentally exposed offspring were homogenized and filtered into single cell suspensions. Cells were washed and counted using Cellometer cell counting chamber slides and Cellometer Auto 2000 software (Nexcelom Bioscience, Lawrence, MA). Acridine orange (AO) and propidium iodide (PI) (Sigma Aldrich, St. Louis, MO) were used to stain and count total cells and live cell numbers per sample. Cells were standardized to a concentration of 2 x 10^6 cells/mL in flow cytometry staining buffer and incubated with fluorescence-activated cell sorting (FACS)buffer and anti-CD16/32 antibody along with anti-mouse CD3e-Allophycocyanin (APC), CD4- Fluorescein isothiocyanate (FITC), and either CD8a-Phycoerythrin (PE) or CD25-PE monoclonal antibodies (mAb) (eBioscience, Inc., San Diego, CA). An additional cell sample for each animal was stained with anti-mouse CD45RB-FITC and NK1.1-PE (eBioscience, Inc., San Diego, CA) and samples were allowed to incubate for 30 minutes in the dark at room temperature. Optimal concentrations of the antibodies and reagents were determined in prior experiments as were isotype controls for color compensation. Stained cells were analyzed using an Accuri C6 flow cytometer and software (BD Accuri Cytometers, Ann Arbor MI) and 25,000 events were collected from each sample. Data reported as absolute number of cells was calculated as the percent gated cells multiplied by the number of nucleated cells counted by the Cellometer Auto 2000.

2.2.8 Statistics

Statistical analyses were performed using SAS (SAS Institute, Cary, NC) and GraphPad Prism 5 (GraphPad software, San Diego, CA) statistical analysis software. Repeated-measures, two-way, and three-way analysis of variance (ANOVA) were used to analyze treatment and treatment x gender interactions by trail. Individual post hoc comparisons were made using least squares means t-tests when ANOVA indicated a statistically significant relationship ($p<0.10$ for behavior tests, $p<0.05$ for all other analyzes). All data are presented as mean ± standard error of the mean (SEM) unless otherwise noted. Experimental "N" refers to the number of treated dams, not offspring and all adult offspring in each treatment group were from a different treated dam. Nested (hierarchical) ANOVA was also performed with dam as a nested variable to account for potential litter effects.

2.3. Results

2.3.1 General dam and litter observations

Numbers of male and female offspring per litter and total litter size did not vary with dose (Table 2.1.). The terminal body weights of offspring did not significantly vary by dose at any time point. Similarly, the number of pregnancies, litters delivered, and litters weaned did not statistically differ based on dose.

2.3.2 Barnes maze

Primary latency for males and females during the acquisition stage at all three ages is displayed in Figure 2.1. Significant differences among age groups and between the sexes indicated that they should be analyzed separately. Intra- and intertrial variability was high across ages and between sexes, which is not unusual for this type of behavioral measure. Occasionally, significant differences were apparent between sexes or among ages when individual trials were evaluated. However, these differences did not persist across all trials or by an observably consistent pattern by trial days.

Recall on the Barnes maze task with the escape box removed is reported in Table 2.2. Overall mice exposed to 50 mg/kg made more correct head pokes during the reference memory day than the group exposed to 100 mg/kg or exposed to vehicle control. At all ages at least one BPA exposed dose group had more head pokes than the vehicle control group. In PND60 females, none of the animals that received 100 mg/kg of BPA made a correct head poke during

the reference test resulting in a group mean of 0 head pokes. This was significantly fewer attempts to enter the correct hole than the 50 mg/kg group at PND60 and significantly fewer than the number of attempts made by mice exposed to 100 mg/kg that were tested at PND21 or PND42.

During the reference test, the average time for control mice (mean of 53.8 s) to locate the correct escape hole was significantly longer than mice that had been exposed to 25 mg/kg (mean of 37.7 s) or to 50 mg/kg (mean of 36.8 s). In animals exposed to 50 mg/kg, PND42 mice took more time to locate the correct hole compared to the two other age groups.

Females, on average, made more primary errors than males on the final reference test. PND60 animals made more primary errors than PND42 mice. The vehicle control mice at PND60 made more errors compared to mice exposed to 25 or 50 mg/kg and the other age groups (Table 2). Nested analysis on reference day variables did not yield any interactions to report.

2.3.3 Overall Activity

When evaluated at PND60, males moved a longer distance during a 180 s trial than the PND21 mice (Figure 2.2.A). At PND42, female mice exposed to 100 mg/kg traveled a greater distance than animals exposed to 50 mg/kg at that age (Figure 2.2.B).

2.3.4 Splenic Immunophenotype

Natural Killer Cells. Splenic natural killer (NK) cell numbers are shown in Figure 2.3. Overall, without considering treatment, male spleens had 10.5% more NK cells than females. PND60 mice had more NK cells than mice at PND42. This age difference was most prominent in animals exposed to 100 mg/kg of BPA in both males and females. No dose related differences were noted for either sex or age group.

B Cells. Figure 2.4. shows the total CD45+ cell count for each treatment group. Female offspring averaged 8.6% more CD45+ cells than males (not statistically significant). There was an overall dose difference noted where mice exposed to 25 mg/kg had significantly higher CD45+ cell counts compared to mice exposed to 50 mg/kg.

T Cells. Splenic T cell CD4/CD8 subpopulations are reported in Table 2.3. Within the CD4+CD8+ subpopulation, the vehicle control group had 26.7% more cells than the 100 mg/kg group after multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. Overall animals exposed to 50 mg/kg had lower CD4+CD8- and CD4-CD8- cell populations than animals exposed to 25 mg/kg. Without considering treatment variations, females had 21.9% more CD4+CD8+ than males. Several significant differences were also noted in CD4/CD25 subpopulations (Table 2.4.). PND60 animals had higher numbers of CD4+CD25+ compared to PND42 animals. However, PND60 animals had significantly fewer CD4-CD25+ cells compared to PND42 animals. Females had significantly more CD4-/CD25- cells than the males overall, but no other sex differences were noted in these cell subpopulations.

TABLE 2.1. Reproductive outcomes for dams dosed with BPA via gavage from pairing with males through weaning of offspring.

(mean ± standard deviation)

	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	Not Gavaged
pregnant/ total dosed	9/10	6/10	7/10	8/10	9/10
Litters delivered/pregnant	7/9	5/6	7/7	6/8	8/9
Male per litter	4.2 ± 2.0	3.8 ± 0.8	4.0 ± 1.7	2.4 ± 2.1	3.6 ± 1.3
Females per litter	2.4 ± 1.9	4.2 ± 1.3	2.4 ± 1.1	2.9 ± 1.5	4.1 ± 1.2
Litter size	6.7 ± 2.5	8.0 ± 1.4	6.4 ± 1.7	5.3 ± 3.2	7.8 ± 1.5
Offspring terminal body weight (g)					
PND21	14.2 ± 1.5	13.1 ± 1.8	13.4 ± 1.5	13.2 ± 1.2	12.1 ± 1.5
PND42	20.5 ± 2.7	21.5 ± 3.0	21.4 ± 2.8	19.6 ± 3.3	20.2 ± 1.3
PND60	23.9 ± 3.1	22.9 ± 2.2	23.2 ± 3.7	23.4 ± 1.6	23.3 ± 2.6

Figure 2.1. Primary latency: average amount of time for mice to reach the escape hole during the acquisition phase. Numbers for males are represented in the left column and females on the right. A, B: postnatal day (PND) 21; C, D: PND 42; E, F: PND60. Data are presented as mean ± standard error of the mean.

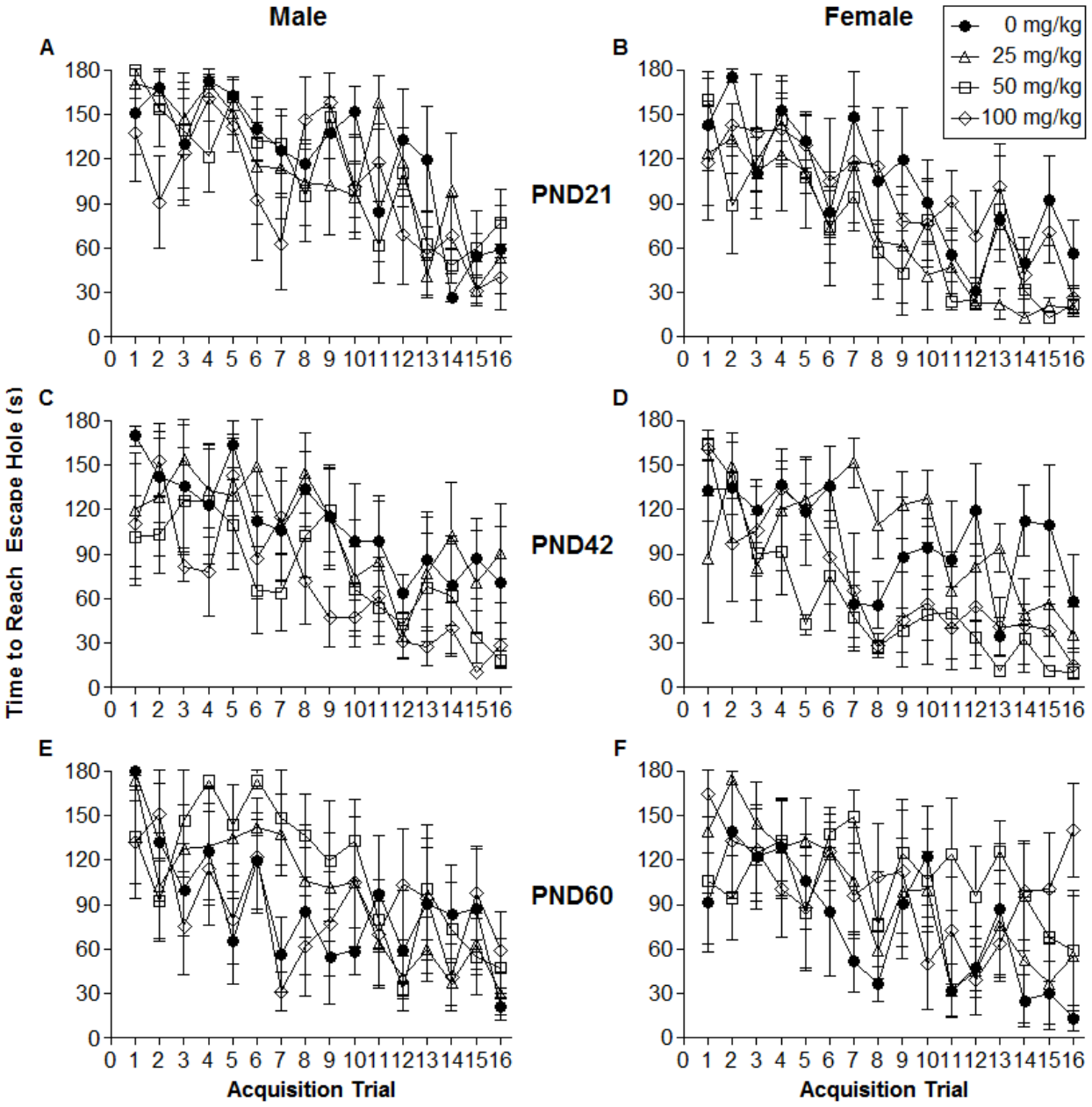

Male
Female
0 mg/kg
25 mg/kg
50 mg/kg
100 mg/kg
A
B
C
D
E
F
PND21
PND42
PND60
Time to Reach Escape Hole (s)
Acquisition Trial
Acquisition Trial

TABLE 2.2. Recall on the Barnes Maze in Adult C57BL/6 Mice Developmentally Treated with BPA. After acquisition training the escape box was removed and number of nose pokes in the correct hole, time to reach the correct hole, and the number of errors prior to reaching the correct hole were recorded for 90 seconds.

Note. Data are reported as the mean ± standard deviation. $p < 0.10$. N=4.

* Significantly higher than other doses

[a] Significantly higher than vehicle control in this age group

[b] Significantly higher than 25 mg/kg in this age group

[c] Significantly higher than 50 mg/kg in this age group

[d] Significantly higher than 100 mg/kg in this age group

† Significantly higher than other age groups

α Significantly lower than other age groups

[e] Significantly higher than same dose at PND21

[f] Significantly higher than same dose at PND42

[g] Significantly higher than same dose at PND60

BPA (mg/kg) to dam	Age	Sex	Correct Attempts to Enter Escape Box	Time to Find Prior Escape Hole	Errors Prior to Reaching Escape Hole
0	PND21	Male	2.0 ± 3.4	51.3 ± 44.8	2.6 ± 1.9
0	PND42	Male	1.3 ± 1.3	63.0 ± 30.6	2.0 ± 2.5
0	PND60	Male	0.8 ± 1.0	48.0 ± 35.3	7.3 ± 11.3[bc]†
25	PND21	Male	1.8 ± 1.3	42.3 ± 32.4	3.5 ± 3.7
25	PND42	Male	4.3 ± 4.8[ac]	36.0 ± 37.4	1.0 ± 0.8
25	PND60	Male	4.3 ± 3.8[a]	16.8 ± 19.2	2.8 ± 1.9
50	PND21	Male	6.0 ± 5.0*†	32.0 ± 26.0	1.0 ± 0.8
50	PND42	Male	0.8 ± 1.0	77.0 ± 22.2[b]†	2.3 ± 2.2
50	PND60	Male	2.8 ± 2.8	41.5 ± 42.8	2.0 ± 2.3
100	PND21	Male	1.0 ± 1.4	60.0 ± 35.4	2.8 ± 2.5
100	PND42	Male	2.3 ± 2.2	44.5 ± 35.5	2.3 ± 3.9
100	PND60	Male	3.3 ± 2.9	41.3 ± 23.4	6.0 ± 3.7
0	PND21	Female	1.8 ± 2.4	46.5 ± 34.8	5.0 ± 4.3
0	PND42	Female	2.8 ± 3.1	52.5 ± 39.0[cd]	4.0 ± 3.6
0	PND60	Female	1.5 ± 1.3	61.3 ± 26.3[b]	1.5 ± 1.9
25	PND21	Female	1.5 ± 1.3	70.0 ± 19.6[c]†	5.3 ± 2.6[c]
25	PND42	Female	1.3 ± 1.9	34.5 ± 37.7	6.3 ± 3.6[d]
25	PND60	Female	2.5 ± 2.5	26.5 ± 42.4	4.3 ± 3.3
50	PND21	Female	6.0 ± 3.4[ab]	18.0 ± 11.0	1.0 ± 1.4
50	PND42	Female	4.0 ± 1.8	17.8 ± 7.1	3.3 ± 1.5
50	PND60	Female	3.5 ± 3.7[d]	34.3 ± 37.8	3.8 ± 3.9
100	PND21	Female	3.3 ± 1.7[g]	43.0 ± 23.3	3.8 ± 1.5
100	PND42	Female	3.0 ± 2.2[g]	10.8 ± 2.1	1.8 ± 1.5
100	PND60	Female	0.0 ± 0.0α	90.0 ± 0.0[bcd]†	7.3 ± 3.4[af]

Figure 2.2. Total distance traveled in an open field (cm) by mice developmentally exposed to BPA over a 180 s trial. A) males B) females. Mean ± standard deviation.

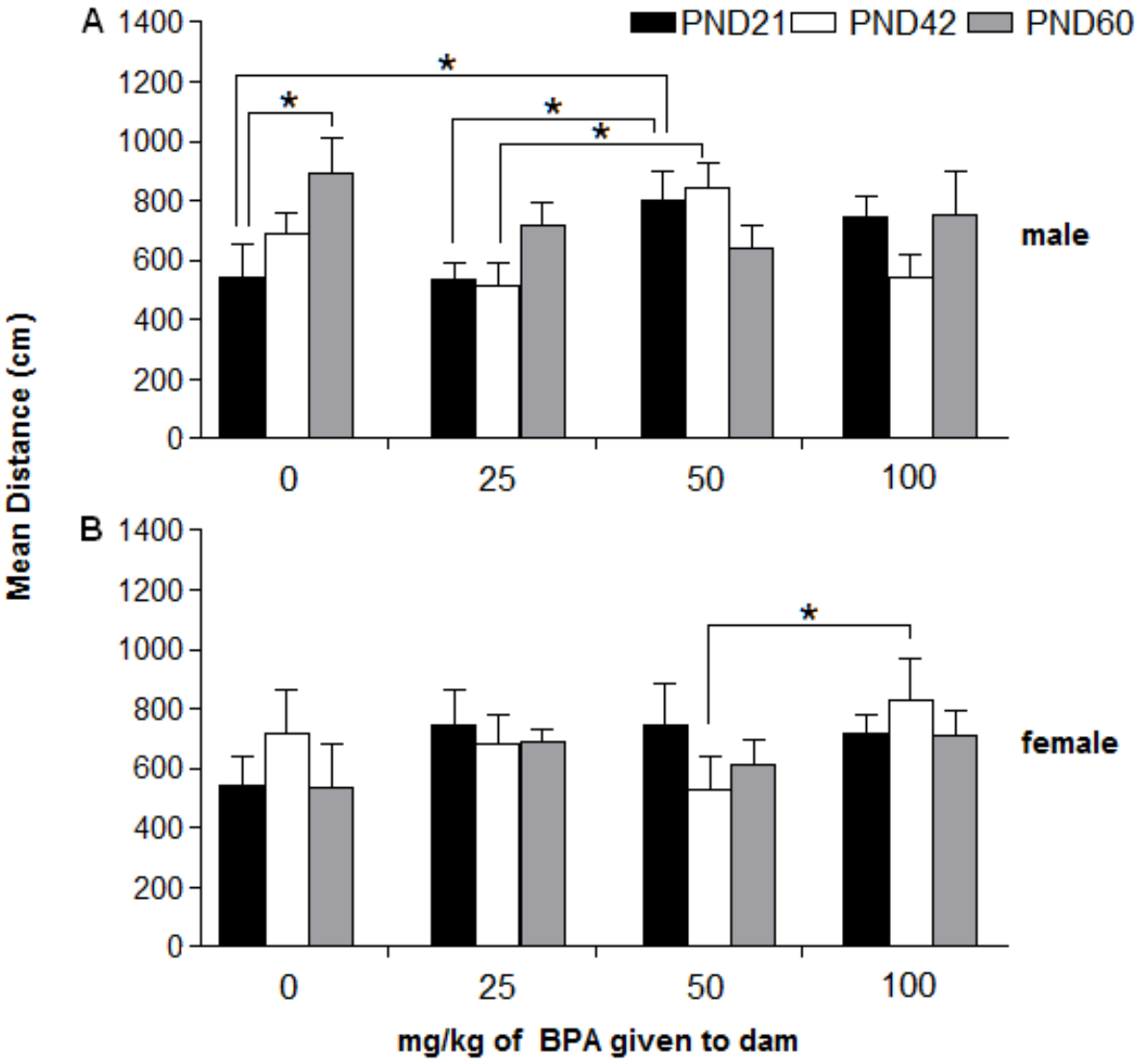

A
B
PND21
PND42
PND60
male
female
Mean Distance (cm)
mg/kg of BPA given to dam
0
25
50
100
200
400
600
800
1000
1200
1400
*

Figure 2.3. Splenic natural killer (NK) cell numbers in offspring exposed to BPA during development.

Mean ± standard error of the mean. N=3-4. A) males B) females. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. No dose effects noted. PND60 animals had significantly higher NK counts compared to PND42 mice at the same given dose.

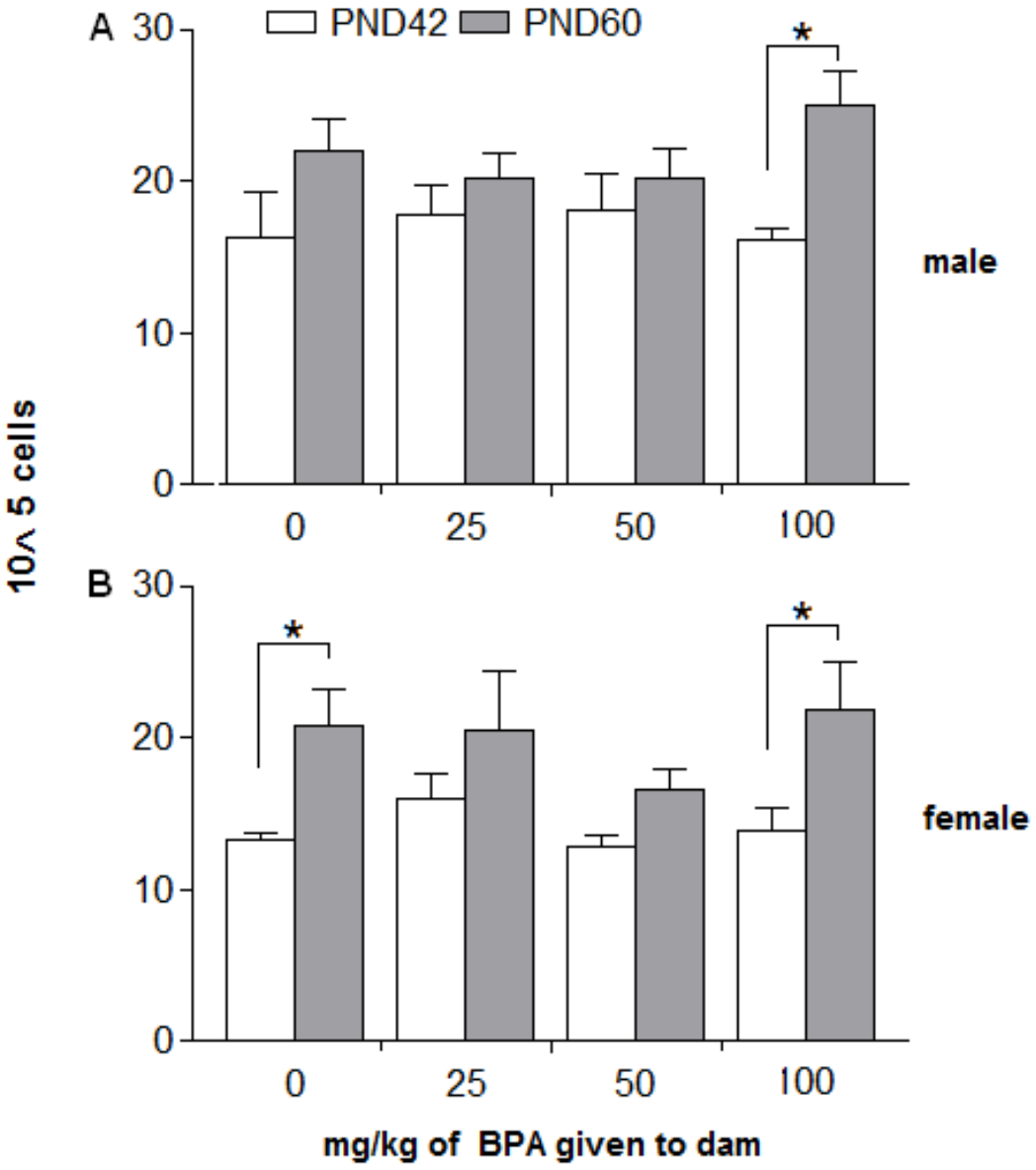

A
B
PND42
PND60
30
20
10
0
*
male
female
10^5 cells
0
25
50
100
mg/kg of BPA given to dam

Figure 2.4. Splenic CD45RB+ cell numbers offspring exposed to BPA during development. A) males B) females. Mean ± standard error of the mean. N=3-4. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen.

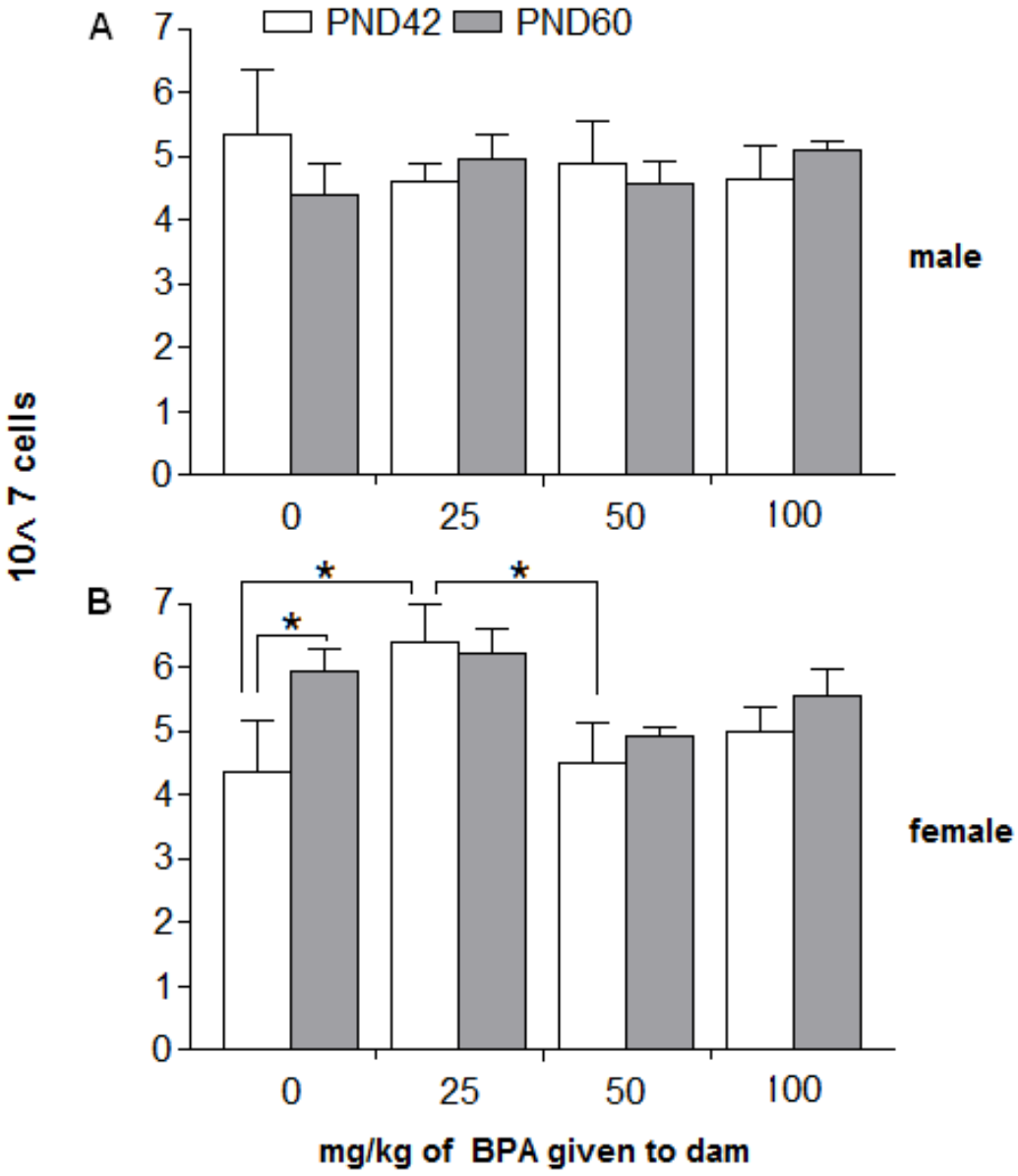

A
PND42
PND60
male
B
female
*
*
*
0
25
50
100
10^7 cells
mg/kg of BPA given to dam

TABLE 2.3. Splenic CD4/CD8 Lymphocyte Subpopulations in Adult C57BL/6 Mice Developmentally Treated with BPA.

Note. Mean ± standard error of the mean. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. $p < 0.05$. N=2-4. All cells reported were gated as CD3+

* Significantly higher than other dose groups at that age.

**Significantly lower than 0 or 25 mg/kg at that age.

† Significantly higher than the other age group

BPA (mg/kg) to dam	Age	Sex	CD4+/CD8- (cells x 10^6)	CD4+/CD8+ (cells x 10^5)	CD4-/CD8+ (cells x 10^6)	CD4-/CD8- (cells x 10^6)
0	PND42	Male	12.2 ± 3.8	2.9 ± 0.6	7.7 ± 0.8	1.5 ± 0.2
0	PND60	Male	11.0 ± 0.7	2.2 ± 0.8	7.2 ± 1.1	1.4 ± 0.3
25	PND42	Male	11.3 ± 1.4	2.5 ± 0.6	6.6 ± 0.8	1.4 ± 0.2
25	PND60	Male	10.6 ± 1.3	3.0 ± 0.6	7.0 ± 0.8	1.6 ± 0.2
50	PND42	Male	11.7 ± 3.1	3.0 ± 0.6	7.2 ± 0.8	1.4 ± 0.2
50	PND60	Male	10.2 ± 1.8	2.4 ± 0.6	7.2 ± 0.9	1.5 ± 0.3
100	PND42	Male	11.8 ± 0.9	3.0 ± 0.6	6.9 ± 0.8	1.5 ± 0.2
100	PND60	Male	12.3 ± 0.5	3.0 ± 0.6	7.7 ± 0.8	1.9 ± 0.2
0	PND42	Female	10.5 ± 4.1	3.6 ± 0.6	6.6 ± 0.8	1.8 ± 0.2
0	PND60	Female	13.7 ± 1.2	5.2 ± 0.6	9.0 ± 0.9†	2.2 ± 0.3
25	PND42	Female	15.5 ± 2.3*	4.1 ± 0.6	9.2 ± 0.8*†	2.5 ± 0.2*
25	PND60	Female	13.6 ± 0.9	4.3 ± 0.6	8.4 ± 0.8	2.6 ± 0.2
50	PND42	Female	10.2 ± 2.6	3.9 ± 0.6	6.0 ± 0.8	1.6 ± 0.2
50	PND60	Female	11.1 ± 0.1	3.1 ± 0.8	7.0 ± 1.1	1.9 ± 0.3
100	PND42	Female	12.1 ± 1.4	2.9 ± 0.6	7.1 ± 0.8	1.7 ± 0.2
100	PND60	Female	12.5 ± 2.3	1.8 ± 0.6**	8.2 ± 0.8	2.0 ± 0.2

TABLE 2.4.

Splenic CD4/CD25 Lymphocyte Subpopulations in Adult C57BL/6 Mice Developmentally Treated with BPA.

Note. Mean ± standard error of the mean. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen.

$p < 0.05$. N=3-4.

All cells reported were gated as CD3+

* Significantly higher than other dose groups at that age.

[a] Significantly higher than vehicle control in this age group

[b] Significantly higher than 25 mg/kg in this age group

[c] Significantly higher than 50 mg/kg in this age group

[d] Significantly higher than 100 mg/kg in this age group

† Significantly higher than the other age group at this dose

BPA (mg/kg) to dam	Age	Sex	CD4+/CD25- (cells x 10^6)	CD4+/CD25+ (cells x 10^5)	CD4-/CD25+ (cells x 10^4)	CD4-/CD25- (cells x 10^6)
0	PND42	Male	12.1 ± 1.2	4.1 ± 0.6	8.3 ± 3.4	8.9 ± 1.0
0	PND60	Male	10.9 ± 1.6	3.1 ± 0.9	4.7 ± 4.8	8.3 ± 1.4
25	PND42	Male	11.5 ± 1.2	3.0 ± 0.6	6.0 ± 3.4	7.8 ± 1.0
25	PND60	Male	10.7 ± 1.1	4.1 ± 0.6	0.4 ± 3.4	8.9 ± 1.0
50	PND42	Male	11.6 ± 1.6	4.5 ± 0.6	13.7 ± 3.4[d]	8.5 ± 1.0
50	PND60	Male	9.7 ± 1.3	4.7 ± 0.7	7.2 ± 3.9	8.4 ± 1.1
100	PND42	Male	11.7 ± 1.2	4.8 ± 0.6[b]	3.5 ± 3.4	8.2 ± 1.0
100	PND60	Male	11.9 ± 1.2	6.3 ± 0.6[ab]	3.8 ± 3.4	9.4 ± 1.0
0	PND42	Female	10.2 ± 1.2	4.0 ± 0.6	7.3 ± 3.4	8.4 ± 1.0
0	PND60	Female	13.2 ± 1.3	4.5 ± 0.7	0.9 ± 3.4	10.7 ± 1.1
25	PND42	Female	15.3 ± 1.2*	3.3 ± 0.6	3.2 ± 3.4	11.2 ± 1.8[ac]
25	PND60	Female	13.3 ± 1.2	5.4 ± 0.6†	0.9 ± 3.4	11.1 ± 1.0
50	PND42	Female	10.1 ± 1.2	4.4 ± 0.6	2.3 ± 3.4	7.7 ± 1.0
50	PND60	Female	11.2 ± 1.6	5.0 ± 0.9	3.3 ± 4.8	9.3 ± 1.4
100	PND42	Female	12.3 ± 1.2	4.2 ± 0.6	3.6 ± 3.4	8.8 ± 0.9
100	PND60	Female	11.3 ± 1.2	7.1 ± 0.6*	1.5 ± 3.4	9.8 ± 1.0

2.4. Discussion

The etiology of developmental disorders remains unclear after decades of research as models for many of these disorders have disparities in the symptoms and outcomes generated compared to the human disease states they are modeling. One of these disorders, autism spectrum disorder (ASD), has been linked to altered immune cell numbers and function. Many theorize that these changes play a role in the etiology in subsets of ASD, but this theory has been difficult to test in existing animal models (Ashwood et al., 2008). Additional data indicates that early-life exposure to environmental toxicants could play a role in animal models of ASD (Hornig et al., 2004). However, environmental toxicant-associated damage or modified endogenous signaling that limits the development of the brain and/or the development of the immune system and would be difficult to discover as deleterious effects would only appear downstream of the initial toxicant damage. The toxicant BPA has been shown to target both immune cells and neurological cells and could mediate the development and function of these cell types (Brinton, 2009; Pierdominici et al., 2010; Huang et al., 2014). This study took the initial steps to uncover this possibility, namely to see if developmental exposure to BPA would jointly affect both the immunophenotype and cognitive performance of an exposed animal.

The objective of this study was to determine if BPA exposure would be associated with alterations of hippocampal dependent spatial learning with concurrent changes to adaptive and innate immune cell populations. Numerous studies have reported that adult or developmental exposure to BPA alters performance on a variety of memory tasks but to date, none have also looked for concurrent changes to the immune system (Eilam-Stock et al., 2012; Diaz Weinstein et al., 2013; Kuwahara et al., 2013).

The Barnes maze is a task that uses a rodent's natural instinct to avoid open areas as motivation to locate an escape point (Barnes, 1979; Barnes et al., 1989; Sunyer et al., 2007). Overall, regardless of age at testing or BPA exposure, after repeated learning trials, all mice had a significant reduction in the time it took to locate the correct escape hole. This outcome suggests that this assessment tool was a reliable method to measure changes to spatial learning. However, all of the mice, including vehicle controls, demonstrated high intra- and intertrial variability across ages and between sexes. While this is not unusual for this type of behavioral measure, additional studies will need to address this issue by increasing sample size in each treatment group to reduce the impacts of this type of variability inherent to this testing paradigm. This variability could be due to handling, tester differences, and proximity of testing to cage changes, or other outside extraneous variables such as levels of noise in the corridor during testing. While all testers were carefully trained and efforts were taken to reduce variability among trials, the results indicate that although all mice learned, individual mice performed very differently across trials and days of testing. Future studies will need to include a much larger sample size to reduce the impact of these factors.

Increased sample size for this study presented a challenge as the Barnes maze is an environmentally-sensitive assessment. The use of multiple mazes to test more mice would not be ideal as it would be difficult to draw conclusions on mice tested in different rooms with different visual stimuli and different handlers. Additionally, this is a time sensitive study that compares mice at specific ages based on date of conception and birth, which is difficult to plan or control. At times during this study, different mice were assessed on the Barnes Maze across 20 or more hours on a given day in order to adhere to the appropriate testing schedule. Future

studies will need to be selective in choosing doses and limiting independent variables so that the same number of dams used in this study could yield higher numbers in future treatment groups.

Differences did emerge among groups on the final day of reference testing. Overall, animals exposed to 50 mg/kg made more correct attempts to enter the escape hole than mice exposed to 100 mg/kg or vehicle controls. At all ages there was at least one BPA exposed group that had significantly more correct attempts than the vehicle control. At PND60, none of the females exposed to 100 mg/kg had a single correct attempt, nor did any of these animals locate the correct hole during the reference test (Table 2.2.). These learning differences could indicate a BPA-mediated alteration in memory formation that led to changes in performance on this task.

Significant age differences were noted in the time it took to initially find the correct escape hole and on the number of primary errors which indicated that as these C57BL/6 mice aged, they responded differently to the same memory challenge. It should be noted that PND60 equates to young adulthood as this strain can live an average of approximately 2 years (PND730) and future research should consider looking at aging effects after developmental BPA exposure.

The open field test was utilized to assess locomotor activity levels to control for changes to motor behavior that could skew performance on the Barnes maze. At PND21 and PND42, male mice developmentally exposed to 50 mg/kg moved significantly farther distances than animals that received 25 mg/kg. At PND42, female mice exposed to 100mg/kg traveled farther in the open field than animals exposed to 50 mg/kg at that age. Males in the vehicle control group demonstrated an age difference where PND60 males moved farther than PND21 mice. Increased locomotor activity could be associated with a reduction in search time and possibly an increase in errors made on the Barnes maze as mice that move more may find the escape hole faster. Several other studies have reported BPA effects on open field assessments though several of

them reported that BPA exposure was associated in a decrease in locomotor behavior (Kubo et al., 2003; Fujimoto et al., 2006; Goncalves et al., 2010; Diaz Weinstein et al., 2013). It should be noted that BPA had not been associated with changes to overall locomotor behavior in pilot work completed in preparation for this study. These differences again could be related to handler effects or other unexpected and uncontrolled variables as they are inconsistent with the literature and our previous findings.

Males have higher NK cell numbers and greater NK cell activity than females in both rodent models and in human studies and our findings were consistent with these published results (Hu et al., 1987; Roberts et al., 2001; Yovel et al., 2001; Klein, 2012). All males in this study, regardless of treatment, had a 10.5% higher splenic NK cell count than their female siblings.

Sex differences in B cell populations were previously reported (Yurino et al., 2004; Fan et al., 2014), but no such differences were observed in our CD45+ cell numbers. Mice exposed to 25 mg/kg had significantly higher CD45+ cell counts compared to mice exposed to 50 mg/kg. This finding is consistent with Yurino et al., (2004) who also noted that BPA can modify B cell numbers and activity.

Male mice in this study had significantly lower numbers of CD4+CD8+ T cells than females. Amadori et al. (1995), reported similar results in human cells where serum CD4+ levels were higher in women than in men. These differences have also been noted in rodent and primate studies where sex hormones regulate CD4+ and CD8+ T cells. Females have higher rates of cytokine signaling associated with these cells relative to males (Roberts et al., 2001; Hewagama et al., 2009; Sankaran-Walters et al., 2013). Our observations for these sub-sets of T cells, like the sex differences noted for NK cells, are consistent with published literature, indicating that BPA exposure alone does not alter expected sex differences in these cell numbers.

Dose-related changes to CD4+ helper T cell populations or functions have been reported previously and our findings were consistent with these results (Lee et al., 2003; Sugita-Konishi et al., 2003). Within the CD4+CD8+ subpopulation, the vehicle control group had 26.7% more cells than the 100 mg/kg group after multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. Mice exposed to 50 mg/kg had lower CD4+CD8- and CD4-CD8- cell populations than animals exposed to 25 mg/kg. Females had 21.9% more CD4+CD8+ cells than males. Several significant differences were also noted in CD4/CD25 subpopulations (Table 2.4.). PND60 animals had higher numbers of CD4+CD25+ compared to PND42 animals and PND60 animals had significantly fewer CD4-CD25+ cells compared to PND42 animals. Females had significantly more CD4-/CD25- cells than did males. Outcomes from BPA exposure in these cell subpopulations suggest that developmental BPA exposure can alter T cell numbers in adulthood. Additional work should follow up on these cell population differences to assess if they these changes in subpopulation numbers would affect clearance rates of pathogens or alter the host response to an immune challenge.

We have shown that developmental BPA exposure can lead to modifications in a variety of immune cell numbers that can differ across different doses and testing time points. This would suggest that further work should be done to explore the nature of these differences. These data suggest that it is not only critical to note the time point of toxicant exposure, but also the timing of the endpoint assessment as we have demonstrated that animals will respond with significantly different behavior based on when they are assessed.

BPA is a widely used chemical incorporated in products that most of us come into contact with on a daily basis. The ubiquitous nature of this chemical could increase the risk of BPA as a developmental toxicant. It is imperative that research models continue to consider the interplay

between the central nervous system and the immune system as this neuro-immune crosstalk could be the instrumental in the discovery and treatment of a host of developmental diseases in the future.

CHAPTER THREE – DEVELOPMENTAL BPA EXPOSURE IN A C57BL/6 MOUSE MODEL INDUCES ALTERATIONS IN SPATIAL MEMORY AFTER LPS CHALLENGE IN ADULTHOOD

3.2. Introduction

Endogenous steroid hormone signaling is pivotal to the timing and duration of development in myriad physiological structures throughout the body, including the central nervous system (Kubo et al., 2001; Brinton, 2009; McEwen et al., 2012). Endocrine disrupting agents that mimic or interfere with this signaling can disrupt the development of an organism. One such chemical, bisphenol A (BPA; 4, 4'-isopropylidene-2-diphenol) was initially used to mimic estrogen in a laboratory setting (Dodds and Lawson, 1936). Now BPA is primarily used in the production of clear and shatter resistant plastics for computers, automobiles, food packaging and in epoxy resins used for lining metal food cans. Approximately five million metric tons of BPA are produced annually to create these products and as a result, this chemical has become a ubiquitous pollutant in our environment (Burridge, 2008).

The main source of human exposure to BPA is through contaminated food and beverages (Kang et al., 2006b; Zalko et al., 2011; Donohue et al., 2013). Heat and acidic or basic conditions used to clean and sanitize these containers can accelerate hydrolysis of the ester bond in BPA monomers, causing leaching of BPA into the food or beverages contained in the plastics (Yu et al., 2011). Despite a short half life of approximately 5.3 hours in humans, BPA has been detected in the urine or serum of more than 90% of those tested in Japan, Canada, Germany, and the United States (Volkel et al., 2002; Calafat et al., 2005; Genuis et al., 2012).

BPA is regarded by the U.S. Environmental Protection Agency (US EPA) as a reproductive, developmental, and systemic toxicant in animal studies (Bisphenol A, 2011). Developmental studies with BPA show that it will cross the placental barrier in mice and other experimental animals and it can be detected in the serum of newborn human infants (Schonfelder et al., 2002; Domoradzki et al., 2004; Nishikawa et al., 2010).

Developmental exposure to BPA was associated with cognitive deficits in working and spatial memory (Carr et al., 2003; Goncalves et al., 2010; Tian et al., 2010; Jašarevic et al., 2011). Tian et al. (2010) demonstrated that BPA exposure during the prenatal and postnatal periods resulted in significantly decreased working memory, as measured on a Y maze, in the exposed offspring. Carr et al. (2003) gave BPA and 17β-estradiol by gavage from postnatal day 1 (PND1) through PND14 to Fischer 344 male and female rats to assess offspring learning and memory on the Morris water maze (MWM) and reported that control animals had a gender-dependent pattern of learning acquisition where males performed better than the female controls (Carr et al., 2003). However, rats exposed to BPA and 17β-estradiol did not have this gender difference (Carr et al., 2003). Carr et al. (2003) hypothesized that BPA can alter gender-dependent patterns of learning.

Jašarević et al. (2011) demonstrated that prenatal exposure to BPA led to a significant decline in spatial memory in male offspring when compared to controls. In this study, male and female deer mice were evaluated with a modified Barnes maze at PND60 and male offspring from control dams performed significantly better than control female offspring and male and female offspring developmentally treated with BPA (Jašarevic et al., 2011). Together these studies demonstrate that BPA can change learning behavior in rodents that are exposed during prenatal and postnatal development.

The objective of this study was to determine if BPA exposure during prenatal and postnatal periods would alter hippocampal dependent performance on a test of spatial learning and memory once the animals reached adulthood PND60. A lipopolysaccharide (LPS) exposure given around the time of learning has been shown to "unmask" developmental deficits in learning and memory (Williamson et al., 2011). Animals were assessed in a "double hit" model where they were developmentally exposed to BPA and then given an acute exposure to LPS around the time of learning. LPS triggers an inflammatory response via induction of the cytokine interleukin-1β that can interfere with memory formation (Bilbo et al., 2005). We hypothesized that exposure to LPS would unmask BPA-induced developmental impacts to hippocampal-dependent learning and memory.

3.3. Materials and Methods

3.3.1 Animals

Adult male (24-26 days old) and nulliparous female C57Bl/6 mice (24-28 days old) were purchased from Charles River Laboratories (Raleigh, NC) and delivered to the East Carolina University (ECU) Brody School of Medicine (BSOM) animal facility (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). Initially, males were housed singly and females were housed four to a cage and were allowed a 14 day acclimation period. After acclimation, males and females were paired into breeder groups with one male and two females per cage. Animals were housed in polycarbonate cages with corn cob bedding, soft bedding material and a hiding tube. All mice were maintained in a 23° ± 3ºC environment with 30-70% relative humidity, and given *ad libitum* access to both food (5P00 Prolab RMH 3000) and water. Animals were maintained in a 12 h light/dark cycle (light, 0730-1930 hours; dark 1930-0730 hours). All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at East Carolina University.

3.3.2 Dosing solution

Dosing solutions were prepared fresh once per week. The appropriate mg/kg concentrations of BPA (Sigma Aldrich, St. Louis, MO, USA) were administered as 0.1 ml of dosing solution per 10 g of body weight. Stock solutions contained 0.04 or 5 mg/ml of BPA that had been sonicated (Branson, 2510) until dissolved into corn oil (Sigma Aldrich, St. Louis, MO, USA).

3.3.3 Treatment

Immediately prior to pairing, all mice were weighed and randomly distributed into dose groups so that no individual group statistically differed by body weight. Starting on the date of pairing with a breeder male, dams received either a vehicle control (corn oil), a low dose (0.4 mg/kg), or a high dose (50 mg/kg) of BPA. Dams were weighed daily and given 0.1 ml of BPA or vehicle control per 10 g of body weight per day via gavage until the weaning of pups on PND21. After a seven day period, males were separated from potentially pregnant mice. Male mice were housed individually and paired back with female mice that did not become pregnant after the initial pairing. After the final pairing, males were removed and euthanized when females reached 24 g in body weight. Female and males that failed to reproduce were also removed from the study and euthanized.

3.3.4 Offspring

On PND1, litters were weighed, sexed and culled to three males and three females per dam. When possible, litters with an insufficient number of male or female pups were backfilled with extra pups of the same age from dams within the same dose group. Offspring were weighed once per week until weaning at PND21. Weaned offspring were housed in same sex groups from their litter when possible (up to three animals per cage). Animals were allowed to remain in these conditions until PND60.

3.3.5 Immune challenge

Adult F1 male and female mice from each dose group were subdivided into two treatment groups; one received an intraperitoneal (i.p.) injection of 0.01 ml sterile saline (Hospira Inc., Lake Forest, IL, USA); another received 0.25 μg/kg LPS (Escherichia Coli 0111:B4; Sigma-Aldrich, Inc., St. Louis, MO, USA) suspended in sterile saline. This injection was given 4 hours prior to starting the acquisition phase of testing on the Barnes maze, on PND63.

3.3.6 Long-term spatial memory

The Barnes maze (Noldus Information Technology, Leesburg, VA, USA) is a 122 cm diameter circular platform raised on a central support to 144 cm from the floor to deter animals from leaping to the ground in efforts to escape. Forty 4.9 cm holes are bored through the platform along the edge and are equally spaced with each other and the outer edge. A black plastic escape box (21.4 cm x 5.4 cm x 8.5 cm) was positioned under one of the 40 holes in the platform and in the same spatial location for the duration of experiments. The surface of the maze is white and brightly lit by overhead lights, which serves as a negative reinforcer to motivate a mouse to find and enter the dark escape box. The hole above the escape box is placed is designated as the "escape hole".

The escape box was removed from the table and repositioned so that it was always in the same location in reference to the visual cues in the room. Four large shapes were cut from construction paper and adhered to the wall for the duration of the experiment. Other objects were carefully noted to make sure they were always in the same place in the room (brooms, trash can,

curtain, and chair). A camera, door, and broom rack were also prominently visible in the room and served as visual cues that a mouse could use to orient themselves when the start box was lifted. The platform and escape box were cleaned after every trial and the table was rotated 90 degrees after every cage was tested.

During the acclimation period that began on PND60, all animals were placed in the center of the table, under an opaque cardboard start box for 10 seconds (s). When the boxed was raised, a mouse was guided to the correct hole and placed in the escape box for two minutes (m). Acquisition began two days after acclimation, which is the learning trial period of the Barnes maze. Mice were placed under the start box in the center of the platform for 10 s, the box was raised, and a mouse was allowed to explore the platform for three m (180 s). After 3 m, or if a mouse entered the escape box, the trial ended and they spent one m in the escape box before being returned to their home cage. If a mouse failed to enter the escape box within 180 s, the observer guided the animal to the escape hole and made sure the animal entered the escape box. During the trial, the amount of time to find the escape hole (latency) and the amount of time until a mouse entered the escape box was measured. The number of primary errors before locating the escape hole and the number of errors after finding the escape hole also were recorded. Acquisition consisted of four days, with at least 15 m rest between each trial.

On the final day of testing a reference test was conducted. The escape box was removed from the platform and a mouse was given a 90 s trial. The number of times a mouse poked its nose into the escape hole, where the escape box had been were recorded. Primary errors, total errors, and latency were also recorded.

3.3.7 Locomotor activity

Overall locomotion was assessed in a clear, 43.18 cm square polypropylene open field chamber where movement was assessed with autotracking software, Autotrack ATM3 4.65 (Columbus Instruments, Columbus, OH, USA). Approximately 15 m after the final test on the Barnes maze, mice were placed in the center of one of four identical open field chambers. Each mouse was allowed three m to explore the open area and the distance traveled was recorded.

3.3.8 Statistics

Initial analyses were performed using SAS (Cary, NC) and GraphPad Prism 5 (GraphPad, San Diego, CA) statistical analysis software and included repeated-measures, two-way, and three-way analysis of variance (ANOVA). Nested ANOVA was also performed with dam as a nested variable to account for potential litter effects. Hypotheses tested included behavioral outcomes as dependent variables and as independent variables, dose combination, repeated measures for dose x trial effects, dose x sex interactions, dose x LPS-injection interaction, and dose x sex x LPS-injection interactions. When test results indicated a statistically significant effect ($p<0.10$), individual post hoc comparisons were made with t-tests.

3.3. Results

3.3.1 General dam and litter observations

Dams exposed to 0.4 mg/kg of BPA had more male than female offspring (69% male) when compared to dams receiving the vehicle control (46% male) (Table 3.1.). Terminal body weight of dams did not significantly vary by dose. Similarly, the number of pregnancies, litter size, litters delivered, and litters weaned did not statistically differ based on dose.

3.3.2 Barnes maze

Primary latency for males and females is displayed in Figure 3.1. Primary latency during the acquisition phase did not differ for males (Figure. 3.1.A). Of the 16 trials, during the acquisition phase, main effect of treatment on primary latency was detected for females on the final trial on day four. The vehicle control females given a LPS injection took significantly more time to locate and enter the escape hole compared to females given saline alone and females exposed to 0.4 or 50 mg/kg of BPA (Figure 3.1.B).

The error rate did not differ significantly for males (Figure 3.2.A) based on developmental BPA exposure or adult exposure to LPS. The error rate for females was significantly different among groups on day 1 where LPS injected animals made fewer primary errors than their saline controls. There was also a dose effect where the animals exposed to 0.4 mg/kg of BPA and given LPS as adults made more primary errors than both the 0 mg/kg and 50 mg/kg animals that also were given an injection of LPS (Figure 3.2.B). These differences were

not observed in saline control animals, but these differences among treatment groups narrowed and then disappeared over the subsequent acquisition days (Figure 3.2.B).

The total time that it took for animals to enter the escape box is presented in Figure 3.3. There was an effect of treatment on total escape time during trial eight for males. Males from the 50 mg/kg BPA group that had saline injected took 32.9% less time to enter the escape box relative to the group injected with LPS and 28.5% less time than the 0.4 mg/kg group that had saline injected (Figure 3.3.A). Additionally, males in the 0.4 mg/kg BPA group that had saline injected and the group with LPS injected took significantly more time than male offspring in the 50 mg/kg exposure group exposed to either saline or LPS during trial ten.

During trials 14 and 16, males in the 50 mg/kg group with saline injected took significantly less time to escape than the paired group with LPS injected. During the final trial of acquisition, male vehicle controls took significantly more time than both of the BPA exposed groups (Figure 3.3.A).

Females also showed significant treatment differences on the total time it took for them to enter the escape box. On day three, during the first trial, both the 0.4 mg/kg and control groups that were injected with saline took less time than the groups injected with LPS. Additionally, animals exposed to the 0.4 mg/kg injected with saline, found and entered the escape box in less time than animals exposed to 50 mg/kg and injected with saline (Figure 3.3.B). On the final day of acquisition, a main effect of treatment was detected on trial four. Vehicle control animals injected with saline took less time, overall, than control animals injected with LPS. In animals injected with LPS, the 50 mg/kg group entered the escape hole in 60.5% less time than the control animals injected with LPS.

Table 3.2. depicts animal behavior during recall on the Barnes maze task with the escape box removed. The number of correct head pokes differed between LPS and saline injected animals, with saline animals averaging 6.0 head pokes into the correct hole during the test and LPS injected animals averaging only 3.2 head pokes into the correct hole. Males exposed to 0.4 mg/kg and injected with saline had significantly fewer correct head pokes (mean of 4.25) than males exposed to 50 mg/kg (mean of 9.25) and injected with saline. Females exposed to 0.4 mg/kg and injected with LPS had significantly more correct head pokes (mean of 6.0) than females exposed to 0 mg/kg and injected with LPS (mean of 1.8) or to 50 mg/kg and injected with LPS (mean of 3.4) (Table 3.2.).

On a test of recall, the time for females (mean of 17.7 s) to locate the correct escape hole was significantly shorter than their male littermates (mean of 33.4 s). The time it took animals to initially locate the correct escape hole differed between LPS and saline injected animals. Saline injected animals took 19.5 s, on average to reach the hole during the test and LPS injected animals took 34.5 s on average. Males exposed to 50 mg/kg and injected with LPS found the escape hole 53.8% faster relative to males exposed to 0.4 mg/kg and injected with LPS. Females exposed to 50 mg/kg and injected with LPS group were 42% faster than the vehicle control group injected with LPS. There were no significant differences found in the number of primary errors made that were associated with sex, LPS injection, or BPA exposure (Table 3.2.). No significant interaction effects were noted for litter or testing order with nested analysis on reference day performance.

3.3 Overall Activity

No significant differences in open field distance traveled were noted in male (Figure 3.4.A) or in female (Figure 3.4.B) mice due to treatment conditions.

TABLE 3.1. Reproductive outcomes for dams dosed with BPA via gavage from pairing with males through weaning of offspring.

Data are listed as mean ± standard deviation

*Indicates a statistical ($P<0.05$) difference from the 0 mg/kg group.

	0 mg/kg BPA	0.4 mg/kg BPA	50 mg/kg BPA
Terminal body weight (g)	28.4 ± 1.9	29.4 ± 1.3	29.0 ± 2.0
Dams pregnant/ dams dosed	9/20	6/20	9/20
Litters delivered/dams pregnant	9/9	4/6	8/9
Male per litter	3.3 ± 1.2	5.0 ± 0.8*	4.5 ± 2.1
Females per litter	3.9 ± 1.3	2.3 ± 1.3*	2.6 ± 1.7
Litter size	7.2 ± 1.0	7.3 ± 1.0	7.1 ± 0.6

Figure 3.1. Primary latency: average amount of time it took to reach the escape hole. Data for 16 trials were pooled into the four days of learning acquisition. Mean ± standard error of the mean. N=2-9. The 0 Saline Male group is not included as there was only an N of 1 that survived for testing in this group.

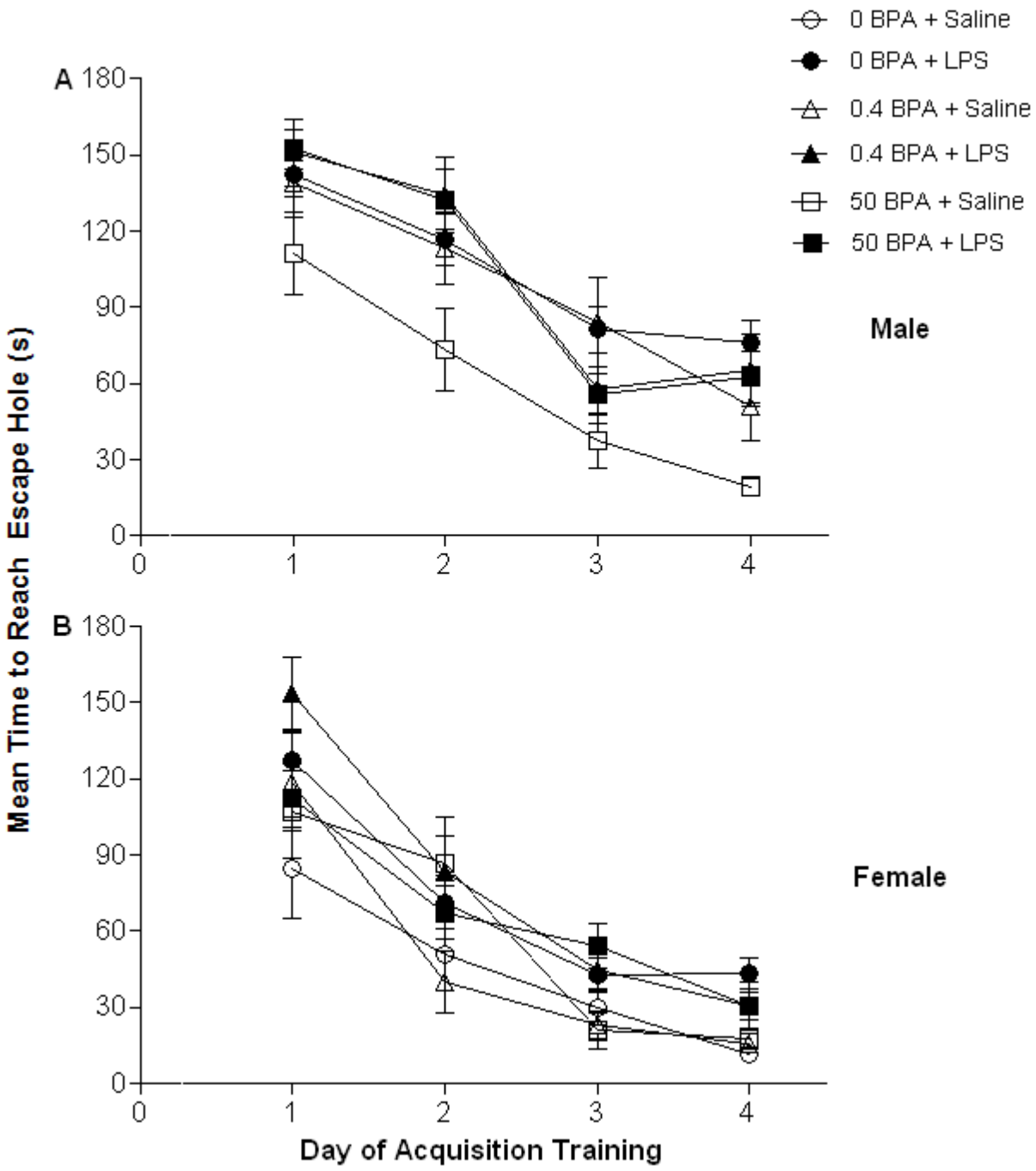

0 BPA + Saline
0 BPA + LPS
0.4 BPA + Saline
0.4 BPA + LPS
50 BPA + Saline
50 BPA + LPS
A
B
Male
Female
Mean Time to Reach Escape Hole (s)
Day of Acquisition Training
0
30
60
90
120
150
180
1
2
3
4

Figure 3.2. Primary errors: Average number of holes explored prior to finding the entrance to the escape box. Data for 16 trials were pooled into the four days of learning acquisition. Mean ± standard error of the mean. N=2-9. The 0 Saline Male group is not included as there was only an N of 1 that survived for testing in this group.

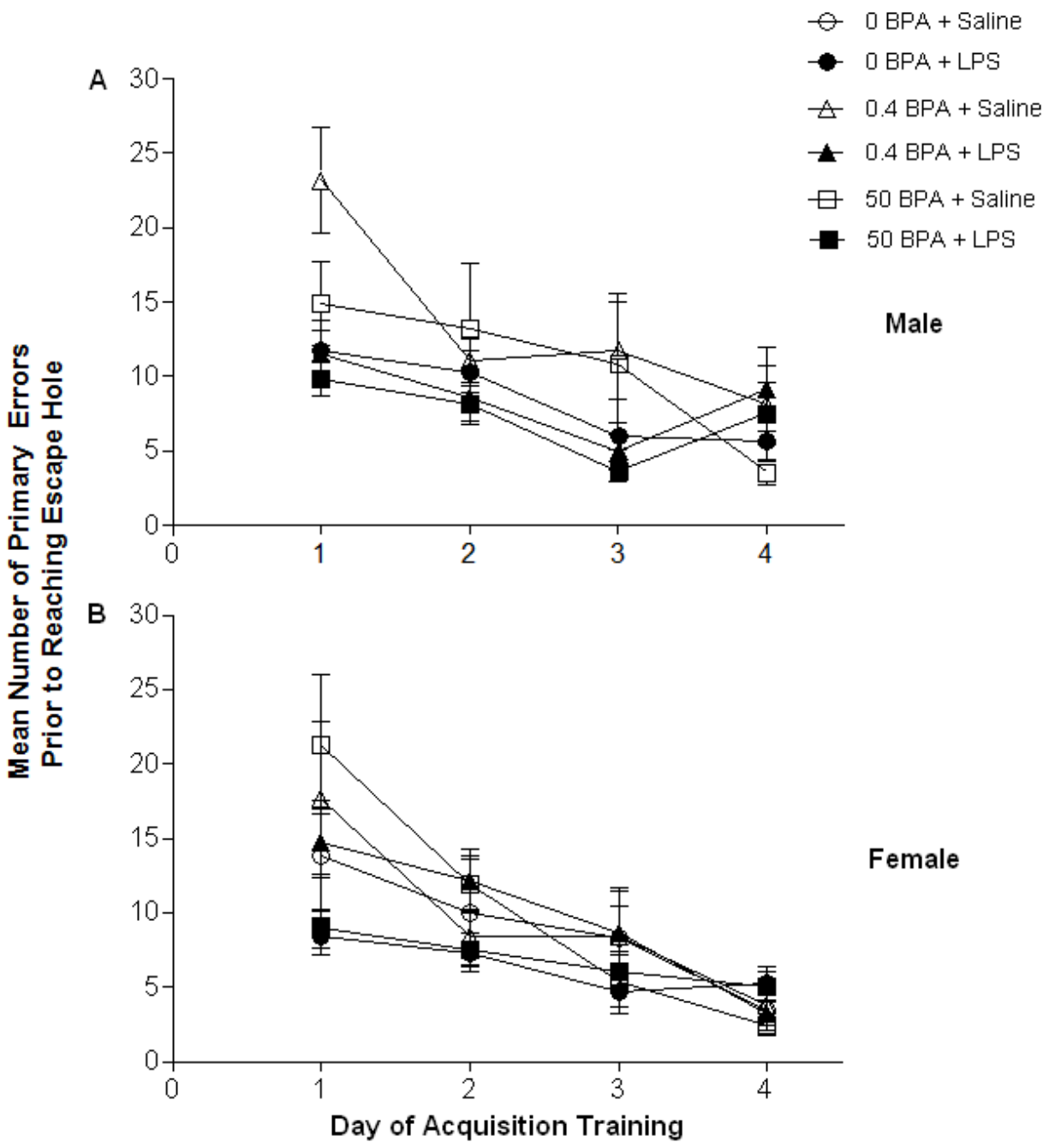

0 BPA + Saline
0 BPA + LPS
0.4 BPA + Saline
0.4 BPA + LPS
50 BPA + Saline
50 BPA + LPS
A
B
Male
Female
Mean Number of Primary Errors
Prior to Reaching Escape Hole
Day of Acquisition Training
0
5
10
15
20
25
30
1
2
3
4

Figure 3.3. Total time (in seconds): Average amount of time that it took for animals to enter the escape box (maximum time allowed was 180 seconds per trial). Data for 16 trials were pooled into the four days of learning acquisition. Mean ± standard error of the mean. N=2-9. The 0 Saline Male group is not included as there was only an N of 1 that survived for testing in this group.

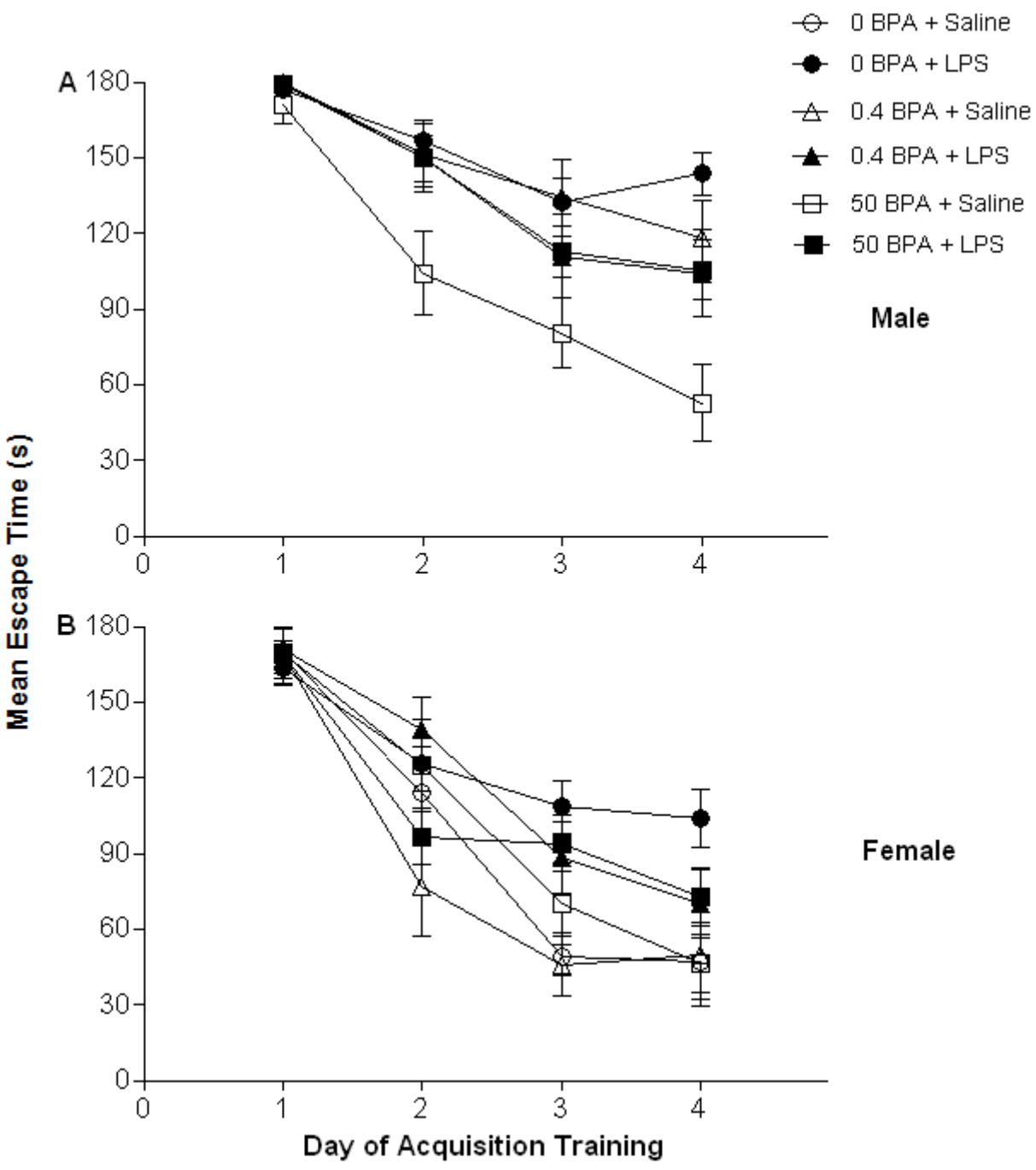

0 BPA + Saline
0 BPA + LPS
0.4 BPA + Saline
0.4 BPA + LPS
50 BPA + Saline
50 BPA + LPS
A
B
Male
Female
Mean Escape Time (s)
Day of Acquisition Training
0
30
60
90
120
150
180
0
1
2
3
4

TABLE 3.2. Recall on the Barnes Maze in Adult C57Bl/6 Mice Developmentally Treated with BPA and LPS.

Note. Data are reported as the mean ± standard deviation. $p < 0.10$. N=2-9. The 0 Saline Male group is not included as there was only an N of 1 that survived for testing in this group.

* Significantly lower than saline control

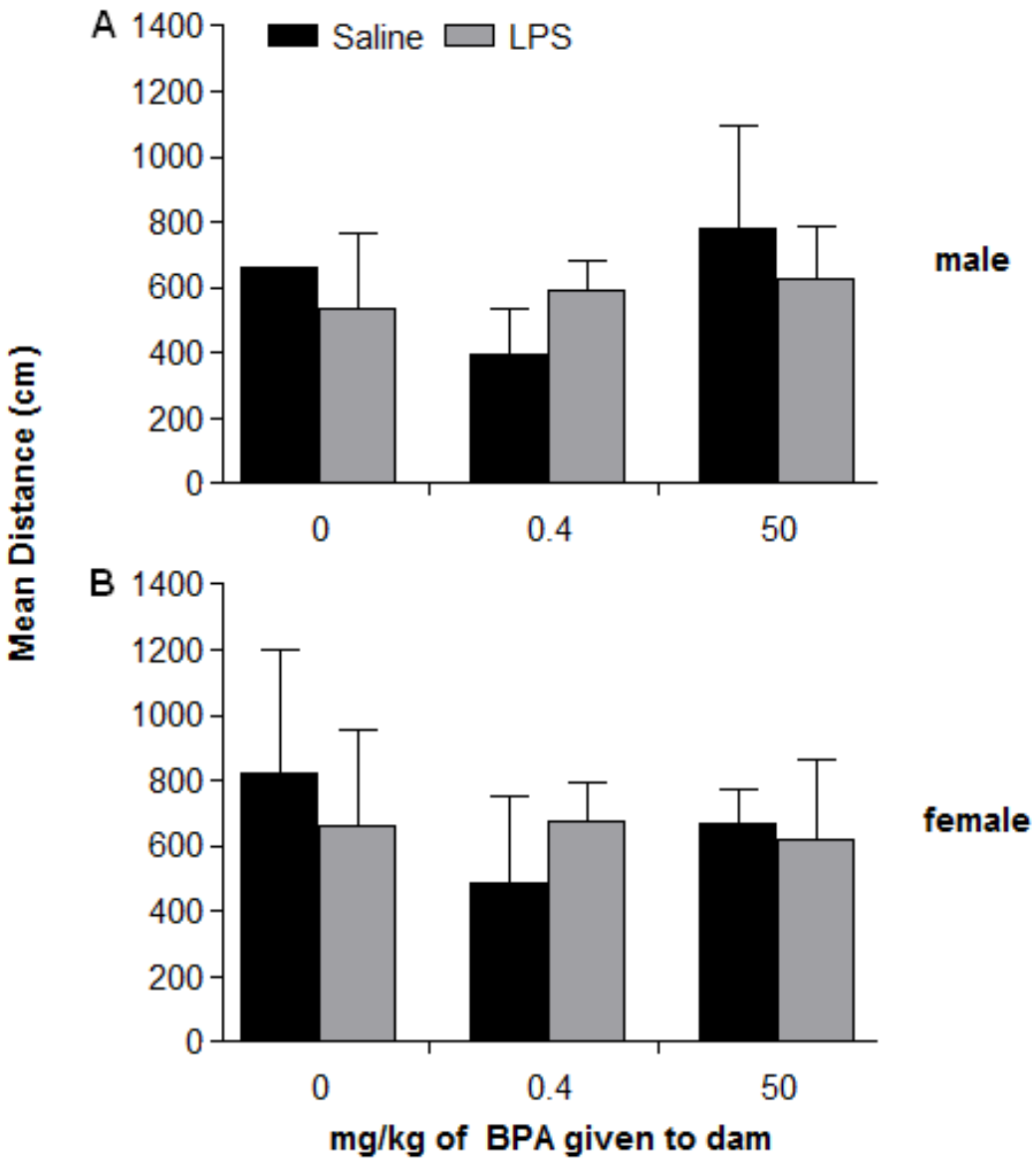
† Significantly higher than other saline exposed BPA dose groups for males

** Significantly higher than other LPS exposed BPA dose group for males

*** Significantly lower than other LPS exposed control group for females

BPA (mg/kg) to dam	Treatment	Gender	Correct Attempts to Enter Escape Box	Time to Find Prior Escape Hole	Errors Prior to Reaching Escape Hole
0	LPS	Male	2.6 ± 1.9	51.9 ± 24.8	4.9 ± 4.5
0.4	Saline	Male	4.3 ± 2.8	39.5 ± 26.5	8.3 ± 6.9
0.4	LPS	Male	2.3 ± 2.3	60.5 ± 29.5**	8.0 ± 6.0
50	Saline	Male	9.3 ± 2.9†	13.0 ± 8.0	4.5 ± 3.8
50	LPS	Male	3.3 ± 2.0*	32.6 ± 16.0	4.0 ± 3.4
0	Saline	Female	6.3 ± 1.6	26.5 ± 18.5	11.7 ± 6.4
0	LPS	Female	1.8 ± 0.9*	35.4 ± 20.1	4.1 ± 5.3
0.4	Saline	Female	6.5 ± 2.5	9.0 ± 1.0	4.5 ± 2.5
0.4	LPS	Female	6.0 ± 2.0	12.8 ± 6.1	2.5 ± 2.3
50	Saline	Female	5.5 ± 2.3	7.8 ± 3.6	3.0 ± 2.5
50	LPS	Female	3.4 ± 1.3	14.9 ± 9.8***	1.8± 1.5

Figure 3.4. Total distance traveled in the open field in cm over a 180 s trial. Mean ± standard deviation. No significant differences were noted for males (A) or females (B) in any treatment group.


A
Saline
LPS
1400
1200
1000
800
600
400
200
0
male
0
0.4
50
B
1400
1200
1000
800
600
400
200
0
female
0
0.4
50
Mean Distance (cm)
mg/kg of BPA given to dam

3.4. Discussion

The endocrine disrupting qualities of BPA exposure during development with regard to reproductive outcomes have been well established (Honma et al., 2002; Salian et al., 2011; Calhoun et al., 2014; Mileva et al., 2014) However, BPA also is reported to induce neurotoxicity (MacLusky et al., 2005; Xu et al., 2013; Yeo et al., 2013). The objective of this study was to determine if BPA exposure would alter hippocampal dependent spatial learning and memory behavior in adult animals exposed during development. This objective was based on studies by other laboratories that demonstrated that adult or developmental exposure to BPA changes performance on a variety of memory tasks including the avoidance of an area associated with foot shock (Goncalves et al., 2010), spatial memory performance in food reward mazes (Kuwahara et al., 2013), performance on a modified Barnes maze (Jašarevic et al., 2011), or changes in sexually dimorphic behavior in a Morris Water maze (Carr et al., 2003; Kuwahara et al., 2013).

We chose the Barnes maze to measure spatial learning and memory of developmentally exposed C57Bl/6 mice. The Barnes maze is an acceptable task for assessing spatial reference memory in mice that uses mild aversive stimuli to reduce confounding behavior caused by increased stress (Sunyer et al., 2007; Brynskikh et al., 2008; Patil et al., 2009; Kennard and Woodruff-Pak, 2011). All of our mice, regardless of exposure had a significant reduction in the time it took to locate the correct escape hole across trials during the acquisition period, these results indicate that this task was a reliable method to determine the impacts of developmental BPA exposure on spatial learning and memory.

Several significant differences were noted during learning trials and on the final reference day between the BPA exposed animals and control animals. During several learning trials, BPA exposed animals took significantly less time to find the correct escape hole when compared to control animals. Additionally, on a test of recall, mice developmentally exposed to BPA made more correct attempts to exit than control animals. These learning differences could indicate a BPA mediated endocrine alteration in memory formation that led to a change in performance on this task. While no clear pattern emerged that would indicate a difference in performance between the 0.4 and the 50 mg/kg exposed animals, it should be noted that at no time did the control animals significantly outperform the BPA exposed animals on any measure taken during this study.

Many studies report that male animals typically have a tendency to perform better than female animals in tests of spatial learning (Berger-Sweeney et al., 1995; Roof and Stein, 1999; Carr et al., 2003; Rajab et al., 2014) However, this sexually dimorphic behavioral difference is not readily apparent in the mouse studies, where males and female animals typically perform at or near the same level (Jonasson, 2005). In this study, female animals located the correct escape hole 88.7% faster than male animals, which is surprising considering what is expected based on the known sex difference in this task, even for mice where the data for sex differences are less compelling. Endocrine disrupting chemicals, including phthalates and BPA, are known to cause a variety of non-reproductive sexual-dependent differences in development, behavior, and disease prevalence (Weiss, 2002). BPA is an estrogen mimicking compound and estrogen plays an integral role in neurological development (Wu et al., 2009). In male mice, an early postnatal surge of testosterone is thought to be required for brain masculinization (McCarthy, 2009). Part of this process involves the conversion of testosterone to estrogen by aromatase enzymes.

Estrogen is therefore the hormone required for brain masculinization. Wu and colleagues (2009) demonstrated that female mice had masculinized brains and behavior after exposure to testosterone or estrogen shortly after birth. If BPA binds to estrogen receptors in the brain during this period of development, this could potentially disrupt endogenous signaling in both the male and female brains and potentially change, "masculinize" or "hypermasculinize" the structures in the brain associated with spatial learning and memory. Although we did not evaluate steroid hormone concentrations, these regions of the brain, or other indicators of sexual development, the results of the Barnes maze task suggest that exposed female animals found the escape hole more rapidly than their similarly-exposed male counterparts. Therefore, BPA exposure induced a sex difference in this behavioral task that was not apparent in male and female control animals and that was opposite to what is commonly observed when male and female animals are compared. In harmony with our results, Xu and colleagues (2010) demonstrated that BPA impaired the spatial memory of male, but not female mice after an 8-week exposure during adolescence.

Williamson and colleagues utilized a model where effects of neonatal bacterial infection were only observed when accompanied by a secondary immune challenge of LPS later in life (Williamson et al., 2011). The neonatal infection caused lifelong changes to the immune system response that could only be seen after LPS challenge. This two hit model was used to demonstrate that the immune system plays an important part in learning and memory. The LPS injection given around the time of learning was shown to "unmask" these developmental deficits in learning and memory (Williamson et al., 2011). In our study, animals also were assessed in a "double hit" model where they were developmentally exposed to BPA and then given an acute exposure to LPS around the time of learning. LPS triggers an inflammatory response, in part, via

induction of the cytokine interleukin-1β (IL--1β), which has been shown to interfere with memory formation (Bilbo et al., 2005). Bilbo and her colleagues demonstrated that neonatal infection with *E. coli* changed cell morphology in the hippocampus and had impacts on memory in the two hit model that incorporated the peripheral LPS injection given around the time of learning (Bilbo et al., 2005). However, the dose used needed to be low, as higher doses of LPS exposure alone have been associated with behavioral changes such as lethargy, reduced eating and fever that can affect memory abilities (Shaw et al., 2001; Rudaya et al., 2005; Sparkman et al., 2005). Therefore, we hypothesized that exposure to a low dose of LPS would unmask BPA-induced developmental impacts to hippocampal-dependent learning and memory. Instead, mice that received saline only were 76.9% faster at finding the correct escape hole relative to mice that received an injected of LPS regardless of BPA dose. Previous studies of the effects of LPS exposure on behavior demonstrated that the resultant production of proinflammatory cytokines (IL-1β, IL-6 and TNFα) exert effects on the hippocampus that disrupt memory formation when given prior to initial learning (Sparkman et al., 2005; Williamson et al., 2011). Although the dose of LPS that we injected was very low (0.25 μg/kg), it may have been sufficient to induce an immune response that produced a difference between the LPS and saline injected animals. Additionally, developmental BPA exposure may alter pathways in the hippocampus that are unaffected by LPS exposure. Future studies with the behavioral task used by Bilbo et al. (2005) and Williamson et al. (2011) will help to determine if the ability of LPS to "unmask" previous developmental deficits is applicable to different toxicants.

To our knowledge, this study is the first to investigate the effects of developmental exposure to BPA on spatial learning performance that concurrently assesses LPS immune effects. This model helps to conceptualize real world toxicant exposure as we are all faced with low level

exposure to a variety of environmental toxicants on a daily basis through polluted air and water. We demonstrate here that developmental exposure to BPA only had mild effects on learning acquisition and did not impair the ability of C57Bl/6 mice in a dose related fashion on the final recall tests. Some evidence from prior studies demonstrated adverse BPA effects on special learning tasks using different animal models or mouse strains (Carr et al., 2003; Goncalves et al., 2010; Xu et al., 2010), whereas other studies did not report BPA induced changes (Ryan and Vandenbergh, 2006; Nakamura et al., 2012). BPA exposure can elicit changes to cells in the hippocampus and other brain regions associated with spatial memory and learning (MacLusky et al., 2005; Nakamura et al., 2006; Hajszan and Leranth, 2010; Eilam-Stock et al., 2012; Inagaki et al., 2012). Future behavioral testing of BPA effects should incorporate tracking software to measure the distance traveled and other behavior parameters to detect more subtle changes to spatial learning such as search strategy or animal mobility as measured by distance traveled during the test. The results of this study suggest that developmental exposure to oral doses of BPA did not compromise the spatial learning ability in this particular experimental model. However, BPA has been reported to pose an endocrine mediated risk during development and should continue to be studied in models that assess its endocrine effects in the presence of other environmentally persistent endocrine mediated neurotoxicants.

CHAPTER FOUR – IMMUNOLOGICAL RESPONSE TO DEVELOPMENTAL BPA EXPOSURE IN A C57BL/6 MOUSE MODEL AFTER LPS CHALLENGE IN ADULTHOOD

4.1 Introduction

The development and functionality of the immune system is regulated by endogenous steroid hormone signaling (Weinstein et al., 1984; Nalbandian and Kovats, 2005; Klein et al., 2010). Estrogen receptors are found on numerous lymphocytes including T cells, natural killer (NK) cells, and B cells (Pierdominici et al., 2010; Huang et al., 2014). Endocrine agents that mimic or interfere with endogenous signaling to these receptors could disrupt the development of a healthy immune system (Goto et al., 2007; Guo et al., 2010; Roy et al., 2012). Bisphenol A (BPA; 4, 4'-isopropylidene-2-diphenol) is one of the most widely studied endocrine disrupting chemicals and will bind to estrogen receptors and disrupt endogenous hormone signaling (Kuiper et al., 1998; Wetherill et al., 2007; Yu et al., 2011).

The majority of human exposure to BPA is widely held to occur through the ingestion of contaminated food and beverages (Kang et al., 2006b; Schecter et al., 2010) with secondary dermal exposure to contaminated thermal papers and through dust inhalation (Biedermann et al., 2010; Liao and Kannan, 2011; Loganathan and Kannan, 2011). BPA is readily inactivated by conjugation reactions to form BPA-glucuronide and BPA-sulfate in the human liver; metabolites that do not bind to estrogen receptors (Taylor et al., 2011). Although this chemical also has a short half life of approximately 5 hours, it can be detected in the urine or serum of more than 90% of those tested in developed countries (Volkel et al., 2002; Calafat et al., 2005; Genuis et

al., 2012; Tharp et al., 2012). Developmental studies demonstrate that BPA will cross the placental barrier in mice and other experimental animals and it has been detected in the serum of newborn human infants, which indicates that it can reach and possibly affect development (Ikezuki et al., 2002; Schonfelder et al., 2002; Domoradzki et al., 2004; Nishikawa et al., 2010).

Several studies have reported that developmental exposure to BPA is associated with immune alterations. Yan and colleagues (2008) demonstrated that developmental BPA exposure could induce persistent immunological effects that lasted into adulthood. BALB/c mice dams were given drinking water containing 1, 10, or 100 nM of BPA during pregnancy and lactation. Male offspring that were prenatally dosed with 100 nM of BPA showed a decrease in CD4+CD25+ T cells (Yan et al., 2008). These offspring were then challenged with an infection of *Leishmania major* in their hind paw. Mice developmentally exposed to 100 nM of BPA demonstrated significantly more foot pad swelling and had a significantly limited rise in CD4+CD25+ T cells after infection when compared to control animals (Yan et al., 2008).

Yoshino and co-workers orally exposed DBA/1J dams to 0.3 or 3 mg/kg of BPA for 17 days after the pairing with sires (Yoshino et al., 2004). Offspring were immunized with hen egg lysozyme (HEL) as an immune challenge to evaluate differences among mice developmentally exposed to BPA and control animals that did not receive HEL immunizations. The Th1 cytokine, interferon-γ (IFN-γ) and the Th2 cytokine, interleukin-4 (IL-4) were both increased in BPA-exposed animals in response to HEL when compared to controls (Yoshino et al., 2004). Additionally, expression of splenic lymphocytes were examined and CD3+CD4+ and CD3+CD8+ cells were increased in mice prenatally exposed to BPA. This laboratory reported that the up-regulation of these immune responses was a lifelong change to the immune systems of these animals (Yoshino et al., 2004). Ohshima and colleagues (2007) demonstrated that

developmental BPA exposure in ovalbumin-specific T-cell receptor trans-genic (OVA-TCR-Tg) mice led to diminished numbers of CD4+CD25+FoxP3+ T cells in response to ovalbumin (OVA). Splenic lymphocytes from the BPA exposed offspring produced more IL-4 and IL-13 and less IFN-γ cytokines relative to control offspring (Ohshima et al., 2007). These developmental immunotoxicity studies of BPA indicate that BPA modifies lymphocyte responses to immune challenges in these animals. Exposure to BPA or other endocrine disruptors could alter the development of the immune system and lead to compromised host defenses throughout the life span. Given the complex nature of immune signaling and function, even subtle changes to the natural signaling pathways could reduce ability to fight off infection, or impair the immune system from distinguishing between attacking cells and endogenous cell types (Roy et al., 2012).

The objective of this study was to determine whether or not BPA exposure during prenatal and postnatal periods would alter the innate or adaptive immune cell response to a peripherally injected lipopolysaccharide (LPS) challenge in adulthood. Animals were assessed in a "double hit" model where they were developmentally exposed to BPA and then given an acute exposure to LPS. LPS triggers a pro-inflammatory response via induction of interleukin-1β, TNFα, and regulation of these cytokines by induction of anti-inflammatory IL-10 (Shaw et al., 2001; Henry et al., 2009). LPS induces fever and sickness behaviors including lethargy and decreased social interaction in higher doses (Shaw et al., 2001). We hypothesized that a global immune challenge of low dose LPS in adulthood would unmask BPA-induced developmental alterations to the adaptive and innate immune response to this immune challenge.

4.2. Materials and Methods

4.2.1 Animals

Adult nulliparous female (24-28 days old) and male (24-26 days old) C57Bl/6 mice were purchased from Charles River Laboratories (Raleigh, NC) and delivered to the East Carolina University (ECU) Brody School of Medicine (BSOM) animal facility (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). Males were housed singly and females were housed four to a cage for an initial 14 day acclimation period. After acclimation, males and females were paired into breeder groups with one male and two females per cage. Animals were housed in polycarbonate cages with corn cob bedding, soft bedding material and a hiding tube. All mice were maintained in a 23° ± 3ºC environment with 30-70% relative humidity, and given *ad libitum* access to both food (5P00 Prolab RMH 3000) and water. Animals were maintained in a 12 h light/dark cycle (light, 0730-1930 hours; dark 1930-0730 hours). All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at East Carolina University.

4.2.2 Dosing solution

Dosing solutions were prepared fresh once each week. The appropriate mg/kg concentrations of BPA (Sigma Aldrich, St. Louis, MO, USA) were administered as 0.1 ml of dosing solution per 10 g of body weight. Stock solutions contained 0.04 or 5 mg/ml of BPA that had been sonicated (Branson, 2510) until dissolved into corn oil (Sigma Aldrich, St. Louis, MO, USA).

4.2.3 Treatment

Immediately prior to pairing, all mice were weighed and randomly distributed into dose groups so that no individual group statistically differed by body weight. Starting on the date of pairing with a breeder male, dams received either a vehicle control (corn oil), a low dose (0.4 mg/kg), or a high dose (50 mg/kg) of BPA. Dams were weighed daily and given 0.1 ml of BPA or vehicle control per 10 g of body weight per day via gavage until the weaning of pups on PND21. After a seven day period, males were separated from potentially pregnant mice. Male mice were housed individually and paired back with female mice that did not become pregnant after the initial pairing. After the final pairing, males were removed and euthanized when females reached 24 g in body weight. Female and males that failed to reproduce were removed from the study and euthanized.

4.2.4 Offspring

On PND1, litters were weighed, sexed and culled to three males and three females per dam when possible or litter size was reduced to a maximum of 6 pups. When available, litters with an insufficient number of male or female pups were backfilled with extra pups of the same age from dams within the same dose group. Offspring were weighed once per week until weaning at PND21. Weaned offspring were housed in same sex groups from their litter when possible (up to three animals per cage). Animals were allowed to remain in these conditions until immune challenges at PND42 or during a learning trial starting at PND60.

4.2.5 Immune challenge

Adult F1 male and female mice from each dose group were subdivided into two treatment groups; one received an intraperitoneal (i.p.) injection of 0.01 ml sterile saline (Hospira Inc., Lake Forest, IL, USA); another received 0.25 μg/kg LPS(Escherichia Coli 0111:B4; Sigma-Aldrich, Inc., St. Louis, MO, USA) suspended in sterile saline. For PND 42 animals this injection was given 4 hours prior to euthanasia. The injection was given 4 hours prior to starting behavior testing on PND63 for a different study in older animals. Euthanasia and spleen removal occurred on PND68.

4.2.6 Flow Cytometry

Spleens from developmentally exposed offspring were homogenized and filtered into single cell suspensions. Cells were washed and counted using Cellometer cell counting chamber slides and Cellometer Auto 2000 software (Nexcelom Bioscience, Lawrence, MA). Cells were standardized to a concentration of 2 x 10^6 cells/mL in flow cytometry staining buffer and incubated with fluorescence-activated cell sorting (FACS) buffer and anti-CD16/32 antibody along with anti-mouse CD3e- Allophycocyanin (APC), CD4- Fluorescein isothiocyanate (FITC), and either CD8a- Phycoerythrin (PE) or CD25-PE monoclonal antibodies (mAb) (eBioscience, Inc., San Diego, CA). An additional cell sample for each animal was stained with anti-mouse CD45RB-FITC and NK1.1-PE (eBioscience, Inc., San Diego, CA) and samples were allowed to incubate for 30 minutes in the dark at room temperature. Optimal concentrations of the antibodies and reagents were determined in prior experiments as were isotype controls for color compensation. Stained cells were analyzed using an Accuri C6 flow cytometer and software (BD Accuri Cytometers, Ann Arbor MI) and 25,000 events were collected from each sample. Data reported as absolute number of cells is calculated as the percent gated cells multiplied by the number of nucleated cells counted by the Cellometer Auto 2000.

4.2.7 Enzyme-linked immunosorbent assay (ELISA)

Immunoglobulin G (IgG) and Interleukin 4 (IL-4) were measured by ELISA kit (eBioscience, San Diego, CA, USA) following the manufacturer's recommended procedure. In the assay 96-well plates were coated with monoclonal antibody with specificity for IgG or IL-4

and incubated overnight. The coated plates were washed 2 times with wash buffer between steps. Capture specific antibodies were detected using horse radish peroxidase (HRP) conjugated isotype specific anti-mouse antibodies and incubated at room temp for two hours. The developed color was measured by a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The concentration of IgG and IL-4 was determined by the standard curve.

4.2.8 Statistics

Statistical analyses were performed using SAS (SAS Institute, Cary, NC) and GraphPad Prism 5 (GraphPad software, San Diego, CA) statistical analysis software. Analysis of variance (ANOVA) was used to analyze treatment and treatment x gender interactions. Individual post hoc comparisons were made using least squares means t-tests when ANOVA indicated a statistically significant relationship ($p<0.05$). All data are presented as mean ± standard error of the mean (SEM) unless otherwise noted. Experimental "N" refers to the number of treated dams, not offspring and all adult offspring in each treatment group were from a different treated dam. Nested (hierarchical) ANOVA was also performed with dam as a nested variable to account for potential litter effects.

4.3. Results

4.3.1 General dam and litter observations

Dams exposed to 0.4 mg/kg of BPA gave birth to more male offspring (69% male) when compared to dams receiving the vehicle control (46% male) (Table 4.1.). The terminal body weights of dams that received BPA by gavage daily did not significantly vary by dose. Similarly, the number of pregnancies, litter size, litters delivered, and litters weaned did not statistically differ based on dose.

4.3.2 Splenic Immunophenotype

Natural Killer Cells. Splenic natural killer (NK) cell numbers in PND68 offspring are shown in Figure 4.1. Male mice exposed to 0.4 mg/kg of BPA had 53.4% more NK cells after LPS injections relative to vehicle control animals and 43% relative to animals exposed to 50 mg/kg BPA (Figure 4.1.A). These dose related differences in female mice were similar to the differences seen in males. Animals exposed to 0.4 mg/kg had more splenic NK cells after LPS injections relative to either the 0 or 50 mg/kg groups that also received LPS. Overall, male spleens had 18.1% more NK cells than females at PND68. However, none of these differences were noted in PND42 littermates (Figure 4.2.).

B Cells. Figure 3 shows the total CD45+ cell count for each treatment group. Female offspring averaged 16.3% more CD45+ cells than males (not statistically significant). There

were no noted differences based on sex, dose, or LPS injections for CD45+ cells in either PND68 (Figure 4.3.) or PND42 mice (Figure 4.4.).

T Cells. Splenic T cell CD4/CD8 subpopulations (Table 4.2.) did not differ by LPS exposure or BPA dose. However, CD4+/CD8-, CD4+/CD8+ and CD4-/CD8+ cells were increased from 20 to 24% in females relative to their male littermates. No sex-related differences were observed in the CD4-/CD8- cell subpopulation in PND68 animals. Sex differences in cell numbers were not observed in PND42 animals (data not shown). T cell CD4/CD25 subpopulations are reported in Table 4.3. Female animals exposed to 50 mg/kg BPA and then LPS injected had 33.4% less CD4+/CD25+ cells than vehicle controls that also received LPS. Female offspring had significantly more CD4+/CD25- cells than the males overall, but no other sex or dose related differences were noted in these cell subpopulations.

4.3.3 Serum Cytokines

Serum IgG levels were 29.3% lower in males that were exposed to 50 mg/kg of BPA and injected with LPS relative to control mice that had also been injected with LPS (Figure 4.5.). These IgG levels also were significantly lower (34.2%) relative to animals exposed to 0.4 mg/kg. These dose differences were not noted in males that had received saline injections or in any of the female mice.

IL-4 was also measured in serum. Female mice exposed to 50 mg/kg and injected with LPS mg/kg had significantly higher levels of IL-4 than females exposed to 0.4 mg/kg (56.5%) or to 0 mg/kg (70.0%) that had also received LPS injections (Figure 4.6.). No other sex or dose related differences were noted in IL-4 levels.

TABLE 4.1 Reproductive outcomes (mean ± standard deviation) for dams dosed with BPA via gavage from pairing with males through weaning of offspring.

*Indicates a statistical ($P<0.05$) difference from the 0 mg/kg group.

	0 mg/kg BPA	0.4 mg/kg BPA	50 mg/kg BPA
Terminal body weight (g)	28.4 ± 1.9	29.4 ± 1.3	29.0 ± 2.0
Dams pregnant/ dams dosed	9/20	6/20	9/20
Litters delivered/dams pregnant	9/9	4/6	8/9
Males per litter	3.3 ± 1.2	5.0 ± 0.8*	4.5 ± 2.1
Females per litter	3.9 ± 1.3	2.3 ± 1.3*	2.6 ± 1.7
Litter size	7.2 ± 1.0	7.3 ± 1.0	7.1 ± 0.6

Figure 4.1. Splenic natural killer (NK) cell numbers in PND68 offspring exposed to BPA during development.

Mean ± standard error of the mean. N=2-9. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. Both male and female animals exposed to 0.4 mg/kg of BPA had higher NK cell numbers in response to LPS injections when compared to animals that received 0 or 50 mg/kg. The 0 mg/kg BPA, saline injected male group was not included as it had N = 1.

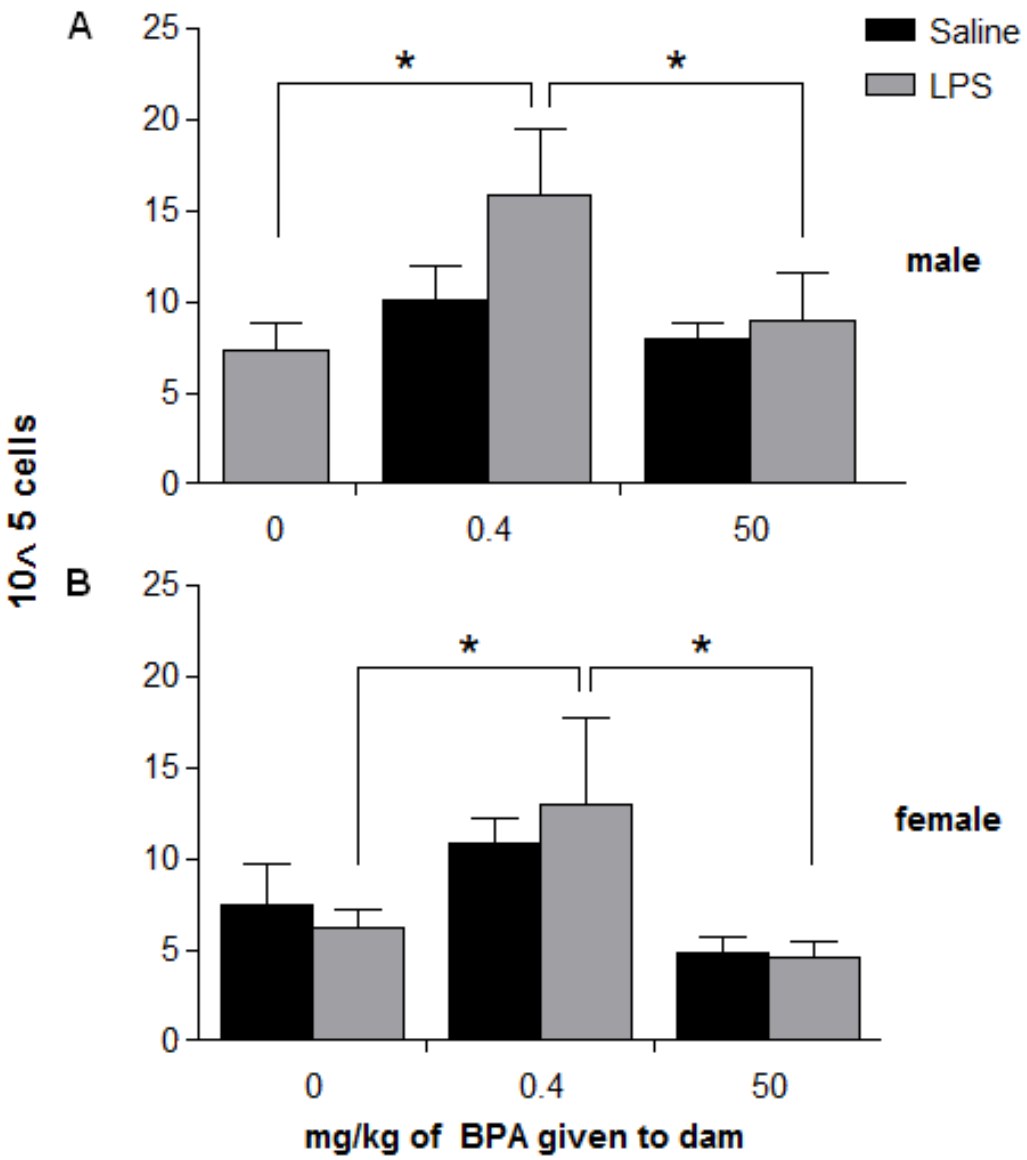

A
B
Saline
LPS
male
female
*
*
*
*
25
20
15
10
5
0
0
0.4
50
10^5 cells
mg/kg of BPA given to dam

Figure 4.2. Splenic natural killer (NK) cell numbers in PND42 offspring exposed to BPA during development.

N=5-6. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. The 0.4 mg/kg group was not included as it had N = 1. This age group of mice received LPS injections 4 hours prior to euthanasia.

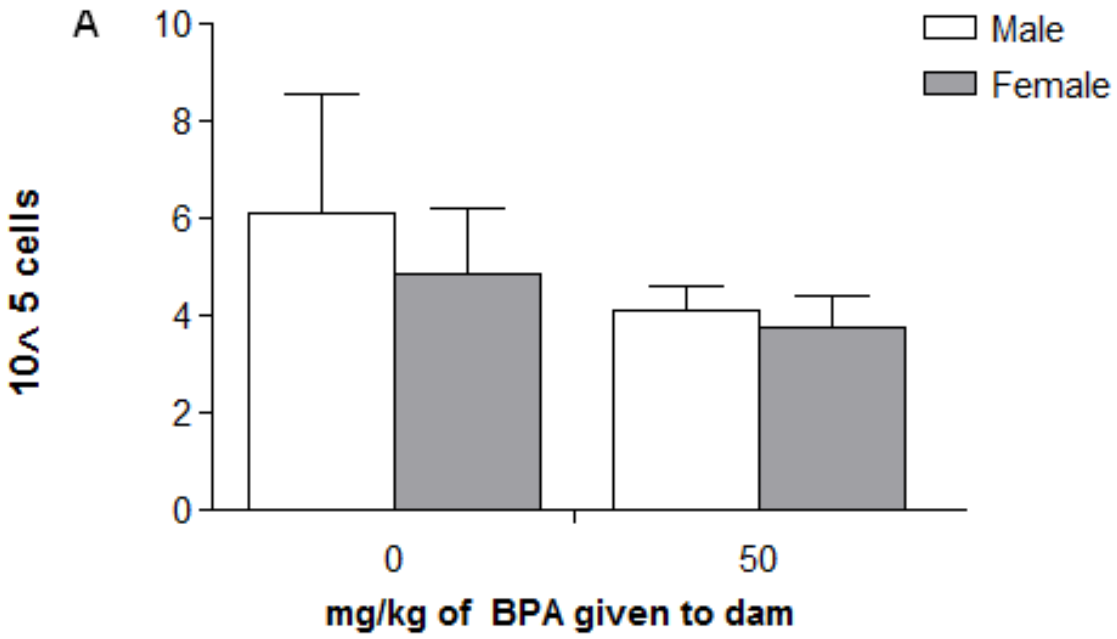

A
10
8
6
4
2
0
Male
Female
10^ 5 cells
0
50
mg/kg of BPA given to dam

Figure 4.3. Splenic CD45RB+ cell numbers from PND68 offspring exposed to BPA during development.

Mean ± standard error of the mean. N=2-9. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. The 0 mg/kg BPA, saline injected male group was not included as it had N = 1.

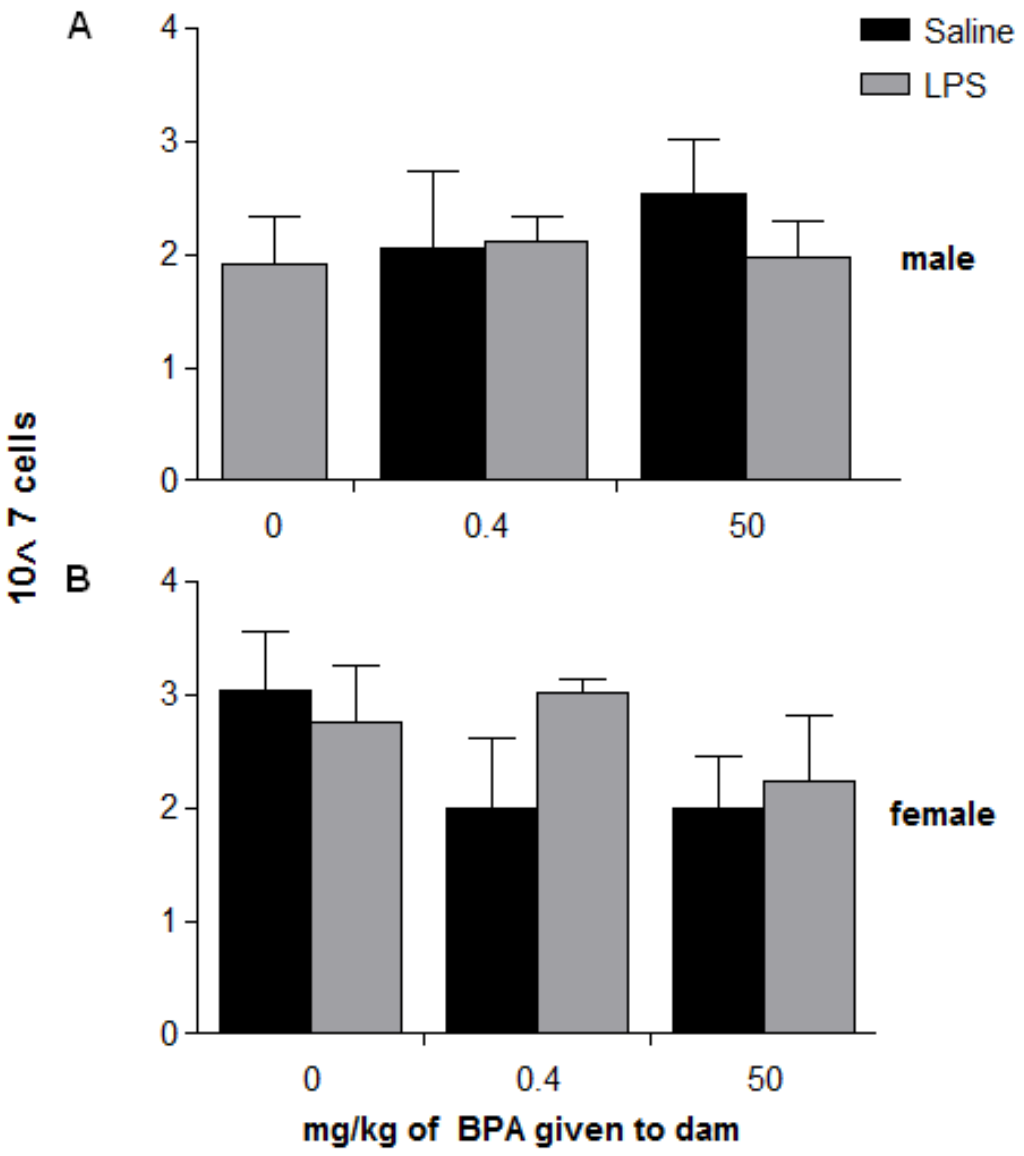

A
B
Saline
LPS
male
female
10^ 7 cells
0
1
2
3
4
0
0.4
50
mg/kg of BPA given to dam

Figure 4.4. Splenic CD45RB+ cell numbers from PND42 offspring exposed to BPA during development.

Mean ± standard error of the mean. N=5-6. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. The 0.4 mg/kg group was not included as it had N = 1. All of these animals received LPS injections 4 hours prior to euthanasia.

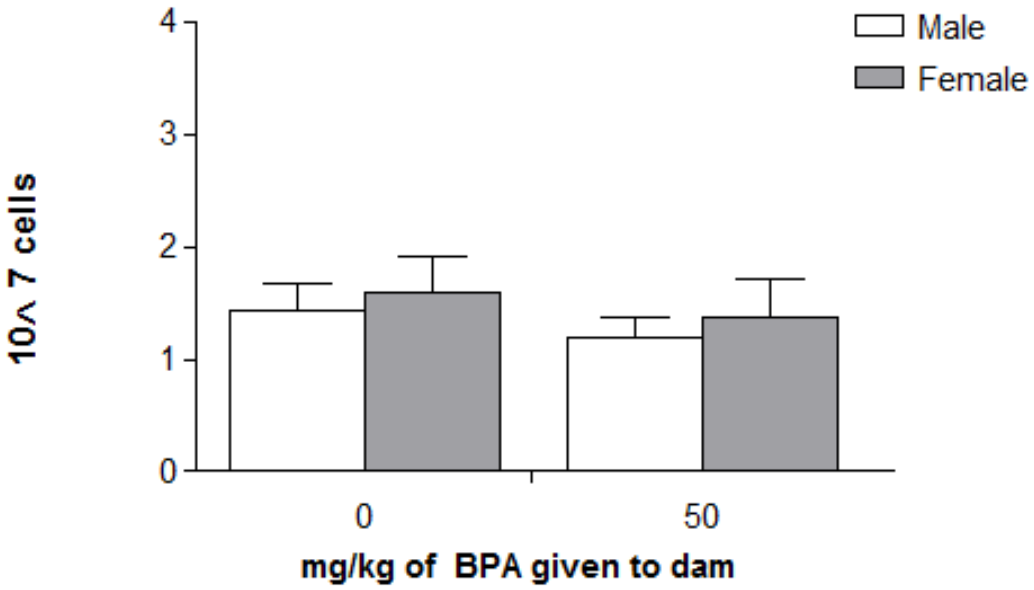

Male
Female
10^ 7 cells
4
3
2
1
0
0
50
mg/kg of BPA given to dam

TABLE 4.2. Splenic CD4/CD8 Lymphocyte Subpopulations in Adult PND68 C57Bl/6 Mice Developmentally Treated with BPA and LPS.

Note. Data are reported as mean ± standard error of the mean. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. $p < 0.05$. N=2-9. The 0 mg/kg BPA, saline injected male group was not included as it had N = 1. All cells reported were gated as CD3+

BPA (mg/kg) to dam	Treatment	Sex	CD4+/CD8- (cells x 10^6)	CD4+/CD8+ (cells x 10^5)	CD4-/CD8+ (cells x 10^6)	CD4-/CD8- (cells x 10^7)
0	LPS	Male	7.4 ± 1.1	4.2 ± 0.9	5.6 ± 0.8	3.3 ± 0.5
0.4	Saline	Male	8.4 ± 2.0	2.8 ± 1.5	6.3 ± 1.4	3.2 ± 0.9
0.4	LPS	Male	7.6 ± 2.0	2.6 ± 1.5	5.9 ± 1.4	3.1 ± 0.9
50	Saline	Male	9.1 ± 1.7	4.5 ± 1.3	5.9 ± 1.2	3.4 ± 0.8
50	LPS	Male	6.8 ± 1.3	3.6 ± 1.0	5.2 ± 0.9	3.2 ± 0.6
0	Saline	Female	10.2 ± 2.0	6.9 ± 1.5	6.9 ± 1.4	3.8 ± 0.9
0	LPS	Female	10.6 ± 1.3	6.0 ± 1.0	7.6 ± 0.9	4.3 ± 0.6
0.4	Saline	Female	11.8 ± 3.4	3.2 ± 2.6	8.1 ± 2.4	4.4 ± 1.6
0.4	LPS	Female	11.4 ± 2.0	3.7 ± 1.5	8.5 ± 1.4	4.3 ± 0.9
50	Saline	Female	8.8 ± 1.7	5.3 ± 1.3	5.8 ± 1.2	3.5 ± 0.8
50	LPS	Female	8.6 ± 1.3	5.4 ± 1.0	6.3 ± 0.9	3.9 ± 0.6

TABLE 4.3. Splenic CD4/CD25 Lymphocyte Subpopulations in Adult PND68 C57Bl/6 Mice Developmentally Treated with BPA and LPS.

Note. Data are reported as mean ± standard error of the mean. Numbers were calculated by multiplying the percent of gated cells by the total number of nucleated cells counted in the spleen. $p < 0.05$. N=2-9. The 0 mg/kg BPA, saline injected male group was not included as it had N = 1.All cells reported were gated as CD3+

*Significantly lower than LPS exposed control group for females

BPA (mg/kg) to dam	Treatment	Sex	CD4+/CD25- (cells x 10^6)	CD4+/CD25+ (cells x 10^5)	CD4-/CD25+ (cells x 10^4)	CD4-/CD25- (cells x 10^7)
0	LPS	Male	7.4 ± 1.2	4.0 ± 0.5	6.6 ± 1.5	3.9 ± 0.6
0.4	Saline	Male	8.1 ± 1.8	2.3 ± 0.8	4.8 ± 2.2	3.7 ± 0.9
0.4	LPS	Male	7.5 ± 1.8	2.6 ± 0.8	5.1 ± 2.2	3.7 ± 0.9
50	Saline	Male	8.5 ± 1.8	2.8 ± 0.8	6.3 ± 2.2	4.1 ± 0.9
50	LPS	Male	7.1 ± 1.4	3.1 ± 0.6	4.9 ± 1.7	3.7 ± 0.7
0	Saline	Female	9.7 ± 2.1	3.6 ± 0.9	5.0 ± 2.6	4.5 ± 1.0
0	LPS	Female	10.7 ± 1.4	5.3 ± 0.6	8.2 ± 1.7	5.1 ± 0.7
0.4	Saline	Female	9.1 ± 2.5	3.6 ± 1.1	5.2 ± 3.2	4.0 ± 1.2
0.4	LPS	Female	11.4 ± 2.1	3.6 ± 0.9	7.8 ± 2.6	5.1 ± 1.0
50	Saline	Female	9.2 ± 1.8	3.1 ± 0.8	6.0 ± 2.2	4.1 ± 0.9
50	LPS	Female	8.9 ± 1.3	3.5 ± 0.6*	7.7 ± 1.6	4.3 ± 0.6

Figure 4.5. Total serum IgG levels in PND68 offspring exposed to BPA during development. Mean ± standard error of the mean. N=2-4. Males exposed to 50 mg/kg of BPA had lower serum IgG after LPS injections when compared to animals that received 0 or 0.4 mg/kg. The 0 mg/kg BPA, saline injected male group was not included as it had N = 1.

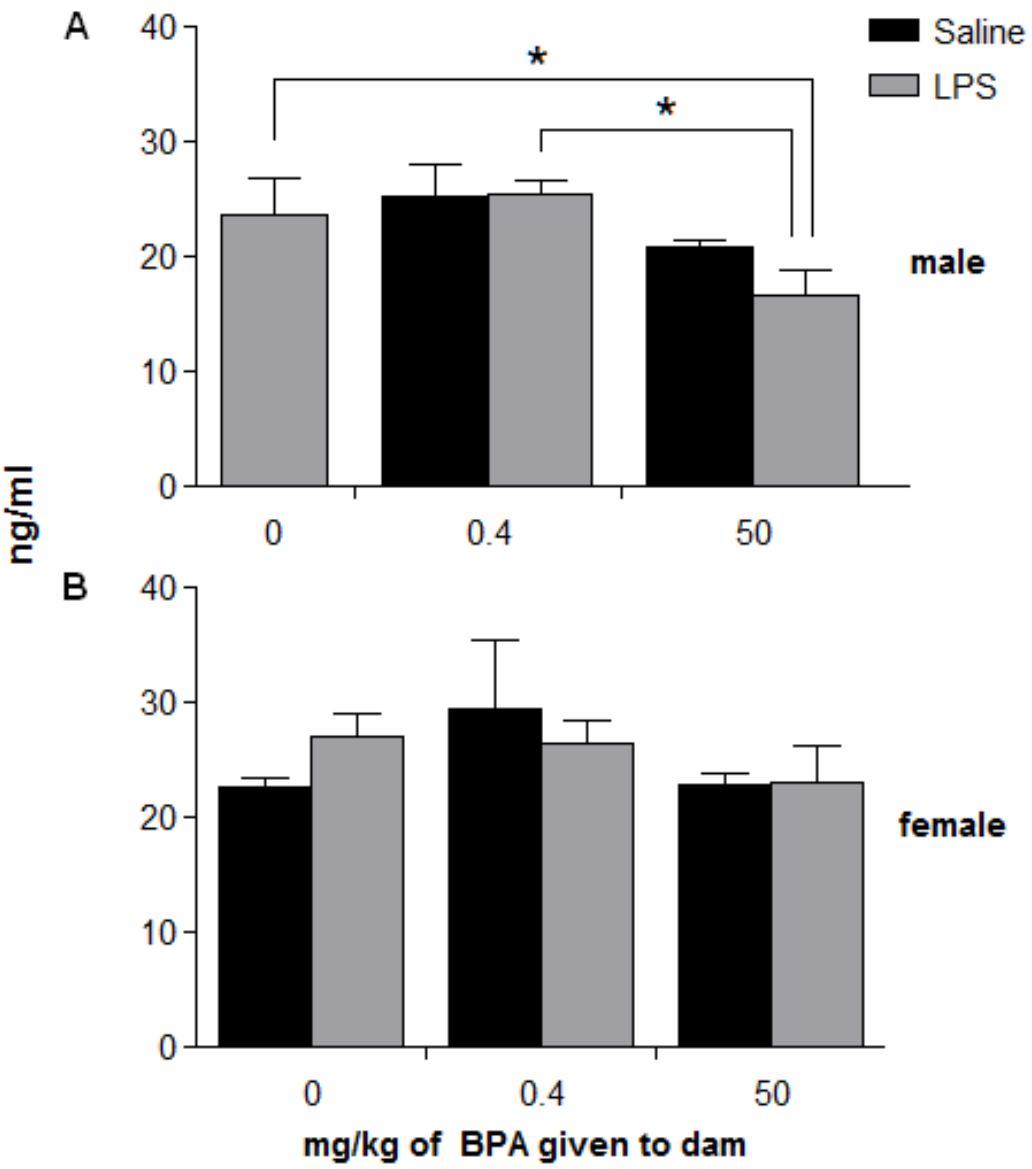
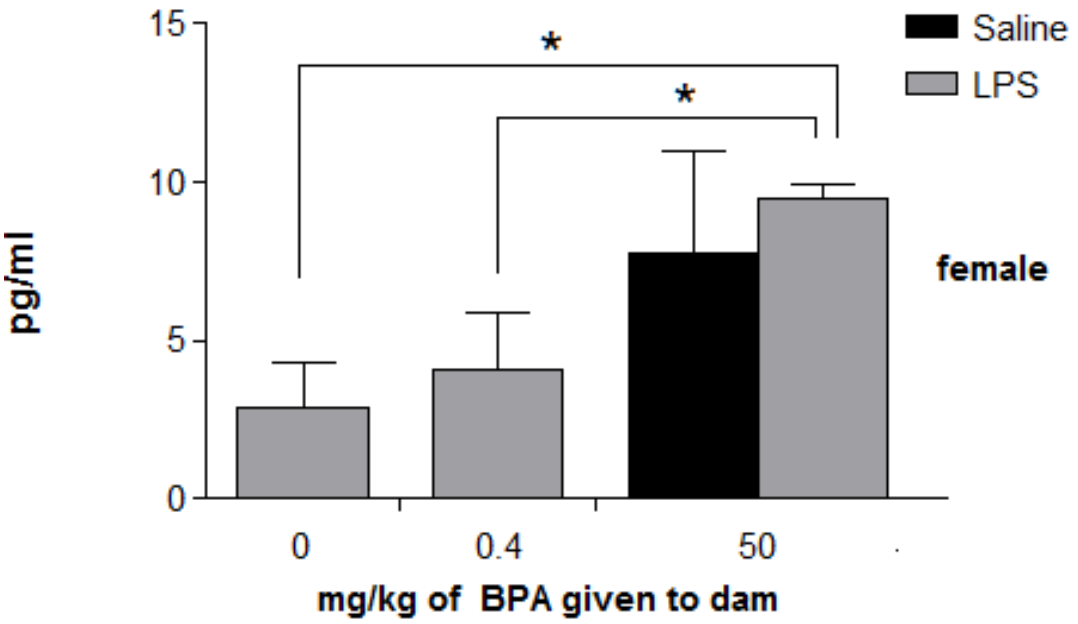

A
B
40
30
20
10
0
ng/ml
Saline
LPS
*
*
male
female
0
0.4
50
mg/kg of BPA given to dam

Figure 4.6. Total serum IL-4 levels in PND68 offspring exposed to BPA during development. Mean ± standard error of the mean. N=2-4. Female offspring exposed to 50 mg/kg of BPA had lower serum IL-4 after LPS injections when compared to females that received 0 or 0.4 mg/kg. The 0.4 mg/kg group was not included as it had N = 1.


15
10
5
0
pg/ml
*
*
Saline
LPS
female
0
0.4
50
mg/kg of BPA given to dam

4.4. Discussion

In this study, we observed several significant alterations in the immune response of BPA and LPS exposed animals. In brief, we saw significant changes associated with BPA exposure in NK cell numbers, CD4+CD25+ cell numbers for female offspring, and in serum total IgG and IL-4 levels. We also observed several sex differences, with male offspring having greater numbers of NK cells and females having a higher number of CD4+ helper T cells and cytotoxic CD8+ T cells. The objective of this study was to evaluate adaptive and innate immunophenotypic alterations in adult animals that were developmentally exposed to BPA. Our hypothesis was that we would only be able to observe these alterations by challenging the adult immune system with a pathogen. Our hypothesis was built on evidence by Bilbo et al., (2005) that early-life exposure to agents that perturb the developing immune system are more likely to be uncovered when the mature immune system is "asked" to respond to an immunological challenge.

The immune system functions properly when complex combinations of cell signals and cellular actions occur in response to invading foreign substances. Even slight changes to the balance of cellular responses or cytokine signaling could reduce the body's ability to fight infections or induce tissue damage associated with hyperresponsivity (Roy et al., 2012). Signals from the endocrine system regulate the immune system and toxicants that perturb endogenous hormone signaling can alter the ability of the immune system to function throughout the lifespan, ultimately increasing the risk for numerous later-life diseases (Roberts et al., 2001; Klein et al., 2010; Roy et al., 2012; Vom Saal and Welshons, 2014).

Our results indicate that male and female mice developmentally exposed to 0.4 mg/kg of BPA had significantly higher splenic NK cell counts than mice exposed to 50 mg/kg or vehicle

controls. However, this was only observed in animals that were given an LPS challenge as adults at PND 68. NK cells play a vital cytotoxic role in the innate immune system by targeting tumor and pathogen infected cells without the need for specialized antigen markers to identify the infecting organism (Raulet, 2004; Wu et al., 2012). NK cell numbers and activation levels respond to mild stressors and to endogenous hormone signaling during the menstrual cycle (Yovel et al., 2001). Therefore, in our system, exposure to BPA may have interfered with developmental hormone signaling, leading to changes in the NK cellular responses to immune challenges in the next generation of mice.

When considering immunological phenotypes, numerous reported sex differences exist, typically showing that females have a more robust response to infections and a higher incidence of autoimmune diseases (Weinstein et al., 1984; Verthelyi, 2001; Marriott and Huet-Hudson, 2006). This more robust response is linked to higher numbers and greater activity of a variety of different cell types, many of which correspond to the findings in this study. However NK cells are the one cell type that differs from this trend. Males have been shown to have higher NK cell numbers and greater activity than females in both rodent models and in human studies (Hu et al., 1987; Roberts et al., 2001; Yovel et al., 2001; Klein, 2012). All males in this study, regardless of treatment, had higher splenic NK cell counts than their female siblings, which is consistent with published literature. However, exposure to BPA did increase NK cell counts for animals that were exposed to 0.4 mg/kg of BPA compared to the other sex-matched dose groups.

Male mice in this study had significantly lower numbers of CD4+CD8- and CD4-CD8+ T cells than females. This is similar to a report on human cells by Amadori et al. (1995), where serum CD4+ levels were higher in women than in men. These differences have also been noted in rodent and primate studies where sex hormones regulate CD4+ and CD8+ T cells. Females

have higher numbers of these cells and associated cytokine signaling relative to males (Roberts et al., 2001; Hewagama et al., 2009; Sankaran-Walters et al., 2013). Our observations for these sub-sets of T cells, like the sex differences noted for NK cells, are consistent with published literature, indicating that BPA exposure alone does not alter sex expected differences in these cell numbers.

In our study, the female C57BL/6 mouse expressed higher numbers of splenic CD4+ and CD8+ cell types after an LPS challenge, but no dose-related differences associated with BPA expose alone were noted. Others reported dose-related changes to CD4+ helper T cell populations or functions after different immune challenges, but these changes were not present in this study after a LPS challenge (Lee et al., 2003; Sugita-Konishi et al., 2003). Our results indicate that females respond differently to LPS, as expected, but BPA exposure alone did not alter LPS-induced changes to helper or cytotoxic T cell responses later in life.

Female mice exposed to 50 mg/kg of BPA had lower splenic CD4+CD25+ cell counts and higher serum concentrations of IL-4 in response to a LPS challenge compared to females in the vehicle control group. These findings are similar to those reported by Yan and colleagues (2008), who demonstrated that prenatal and adult BPA exposure was associated with a decrease in CD4+CD25+ T cells and a dose-dependent increase in IL-4 levels in male offspring. It should be noted that Yan et al. (2008) only looked at responses of male offspring. BPA exposure was associated with increased IL-4 signaling in a variety of different immune challenge studies (Lee et al., 2003; Tian et al., 2003; Yoshino et al., 2004; Ohshima et al., 2007). These cell and cytokine changes indicate that developmental BPA exposure can alter T cell function in adulthood, thus altering host response to infections and other immune challenges.

We measured a significant decline in serum total IgG in adult male mice exposed to 50 mg/kg of BPA compared to those exposed to 0.4 mg/kg or vehicle controls, but only in males that had received the LPS challenge. IgG should rise in response to LPS challenges; this low concentration of IgG in male mice exposed to 50 mg/kg of BPA could indicate an overall lower immune response in this group, but we were unable to discern if this was associated with a particular cell type.

Differences in B cell populations are correlated with sex (Yurino et al., 2004; Fan et al., 2014), but no such differences were observed in our CD45RB+ cell numbers. BPA has been reported to modify B cell numbers and activity (Yurino et al., 2004) however, similarly to the results of Sugita-Konishi et al. (2003), we did not see BPA dose-related differences in B cell numbers.

By utilizing a double-hit model of developmental BPA exposure combined with a later life immune challenge (i.e., LPS), we were able to assess changes attributable to two challenges in the same animal model. We showed that developmental BPA exposure can lead to modifications in a variety of immune cell responses that can change how organisms respond to an immune challenge later in life. BPA is a widely used chemical and could pose a risk as a developmental toxicant as it is detectable in the serum of 96% of pregnant women (Woodruff et al., 2011). It is imperative that research models include BPA and other endocrine disruptors to determine if exposure impacts bacterial and viral immune responses. We demonstrated that developmental BPA exposure changes how male and female animals respond to an immune challenge in adulthood, which suggests that lifelong changes to the immune system can be induced by early-life exposure to BPA.

CHAPTER FIVE – GENERAL DISSCUSSION AND SUMMARY

The overall objective of this research was to improve our understanding about the impact that developmental BPA exposure has on visuospatial learning behavior and on the general immunophenotype in these same animals. Specifically, this research allowed us to conclude that: a) Developmental BPA exposure was associated with alterations of learning behavior compared to controls. However, these changes were not consistently related to dose and responses changed between sex and the age of the mice when evaluated. b) Exposure to BPA alone did not alter expected sexual dimorphisms in immune cell populations. c) LPS exposure can influence changes to learning on the Barnes maze.

The Barnes maze was utilized as the learning task for mice in all of these studies and our results clearly indicate that as the learning trials progress, the time to find the escape hole shortens. This consistent pattern occurred for almost all of the animals and indicates that we properly utilized this task to evaluate hippocampal-dependent learning and memory. The task is based on the innate nature of the species being tested. Mice and rats prefer darker environments and enclosed areas compared to open, brightly lit areas (Barnes et al., 1989). Prey species will spontaneously search for a way to leave an environment where they are exposed in favor of an enclosed space (Jašarevic et al., 2011). In chapters 2 and 3 we examined the outcomes of BPA exposure on adult offspring that were exposed to BPA during in utero and lactational contact with dams. The performance for all of these animals indicated that they were motivated to search for escape from the circular table top from the first day of testing as mice used most of their time searching around the table top for a way to escape that environment.

The principal objective of this research was to test for impacts of exposure to BPA. Chapter two discusses the learning and immune outcomes of developmental BPA exposure. This study included behavioral results with high intra- and intertrial variability across ages and between sexes. While this is not unusual for this type of behavioral measure, significant differences between ages and dose groups did not conform to any observable patterns during acquisition learning. On the final reference day, exposure to BPA was associated with more correct attempts at entering the escape hole in the Barnes maze. The fewest number of correct attempts was observed in BPA-exposed females at PND60. Several significant differences in behavior were noted among age groups, which indicate that as these C57BL/6 mice aged, they responded differently to the same tasks.

This study included both positive and negative control groups in an effort to provide a control reference for comparisons. The negative control group was a group of mouse dams that were handled and weighed everyday but were never subjected to gavage. This would control for any changes resulting in this technique without regard to the chemical being gavaged. A positive control group was planned to mimic fetal alcohol syndrome to provide an animal model where developmental exposure to a toxicant produced both cognitive and immunological changes for comparison. Dams subjected to ethanol gavage either died, resorbed their litters, or cannibalized their pups so that no positive control offspring survived to undergo behavioral testing or immunophenotyping. Had a positive control group been available, additional germane comparisons could have been made to the BPA offspring endpoints and outcomes which would have strengthened our findings.

Chapters three and four covered experiments where offspring were assessed on a Barnes maze at PND60, beginning 4 hours after a single challenge with LPS. Mice developmentally

exposed to different doses of BPA made more correct escape attempts than vehicle control animals. During this set of experiments, no control groups outperformed BPA exposed groups on any behavioral measure. These results indicate an association between BPA exposure and better memory performance on the Barnes maze in C57BL/6 mice.

Control saline-injected animals significantly outperformed LPS-challenged animals which would indicate that even low dose exposure to LPS could alter learning and behavior with this mouse model on the Barnes maze. We had hoped that dual exposure to BPA and LPS would "unmask" differences among dosing groups, but no clear pattern emerged that would indicate that either 0.4 or 50 mg/kg exposed mice would perform differently to the other group.

In these experiments female animals in general were 88% faster at this visuospatial task than males. This is inconsistent with previously reported studies and our prior findings where males outperform or perform equally with females on this type of evaluation. BPA, as an endocrine disrupting agent could potentially alter endogenous signaling in both the male and female brains and potentially change, "masculinize" or "hypermasculinize" the structures in the brain associated with spatial learning and memory. Although we did not evaluate steroid hormone concentrations, these regions of the brain, or other indicators of sexual development, our results suggest that exposed female animals found the escape hole more rapidly than their similarly-exposed male counterparts. Therefore, BPA exposure induced a sex difference in this behavioral task that was not apparent in male and female control animals and that was opposite to what is commonly observed when male and female animals are compared. BPA exposure could be associated with impaired spatial memory in males on this task. This research demonstrated that BPA could alter learning behavior, but no consistent patterns emerged with regard to the effects of BPA dose or age at testing. Overall, our findings suggest that developmental BPA exposure,

can alter learning and immune cell types but additional research should be conducted to determine which doses cause these alterations.

We also reported significant changes to NK cell numbers, CD4+CD25+ T cell numbers in females and alterations to IgG in males and IL-4 in female mice. BPA exposure led to modified immune cell numbers at different doses and ages. BPA exposure in our research did not alter expected sex differences in immune phenotype. Outcomes from BPA exposure in these cell subpopulations suggest that developmental BPA exposure can alter T cell numbers in adulthood. Additional work should follow up on these cell population differences to assess if these changes in subpopulation numbers would affect clearance rates of pathogens or alter the host response to an immune challenge.

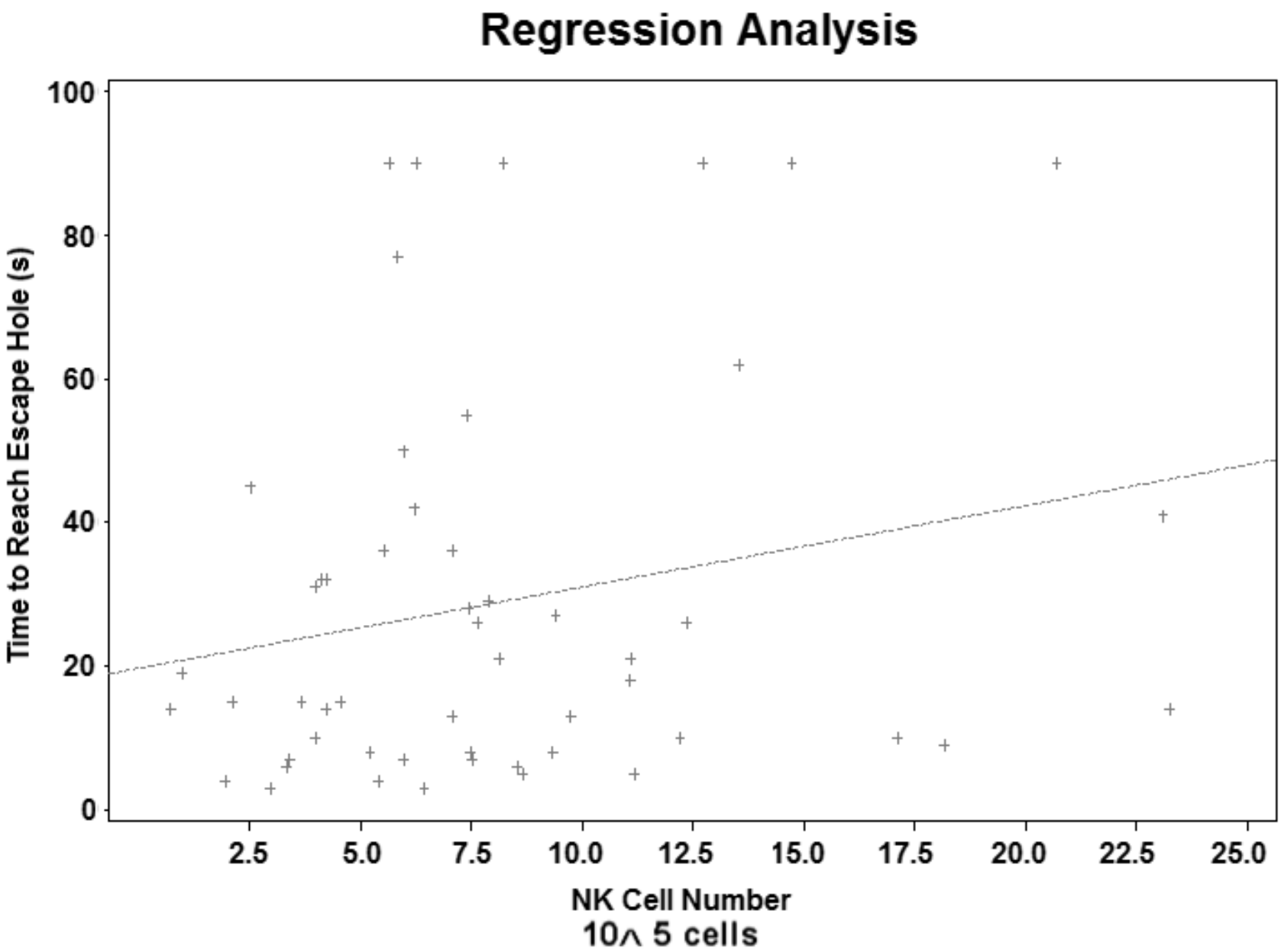
We have shown that developmental BPA exposure leads to modifications in a variety of immune cell numbers that can differ across different doses and testing time points. This would suggest that further work should be done to explore the nature of these differences. These data suggest that it is not only critical to note the time point of toxicant exposure, but also the timing of the endpoint assessment as we have demonstrated that animals may have significantly different immune numbers based on their age.

Future testing should look for correlations between immune and learning outcomes to determine if measurable changes in immune parameters predict learning delays or disability. We ran simple linear regression models individually comparing documented behavior and immune cell numbers in our mice. Using immune variable outcome did not generate any prediction models that would significantly predict behavior variation on either primary latency or the correct number of head pokes recorded on the final day of behavior testing. Figure 5.1 represents the best prediction model generated and demonstrates that the number of NK cells is a

poor predictor of primary latency on the Barnes maze. While there were not any useful models generated, this does not indicate that immune system modifications could not predict or be associated with learning changes, but that this model of comparing changes in C57BL/6 adult mouse immune cell numbers to learning behavior outcomes was ineffective at finding any significant patterns of prediction.

Figure 5.1. Simple Linear Regression Between Primary Latency and NK cell numbers

Note: Primary latency = 19.636 + 113 *10^7 NK cells


Regression Analysis
Time to Reach Escape Hole (s)
100
80
60
40
20
0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
NK Cell Number
10∧ 5 cells

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APPENDIX A

ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER

East Carolina University

Animal Care and
Use Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

June 12, 2013

Jamie DeWitt, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. DeWitt:

The Amendment to your Animal Use Protocol entitled, "Effects of Endocrine Disrupting Compounds on Immune and Nervous System Development in Mice", (AUP #W227) was reviewed by this institution's Animal Care and Use Committee on 6/12/13. The following action was taken by the Committee:

"Approved as amended"

****Please contact Dale Aycock prior to any hazard use**

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure