Effects of endocrine disruption and immune status on social behavior

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B.A. Psychology University of Wisconsin – Madison, 2008

A Dissertation presented to the Graduate Faculty of the University of Virginia in

Candidacy for the Degree of Doctor of Philosophy

Neuroscience Graduate Program

University of Virginia

August 2015

Abstract

Many factors affect behavior and development. Two important examples are immune system function and chemicals in the environment. Endocrine disrupting chemicals are abundant and affect behavior, hormone levels, reproduction, and neurodevelopment. One such chemical, di-(2-ethylhexyl) phthalate (DEHP), is used in hospital equipment, food packaging, flooring, and children's toys. Dose response studies show a non-monotonic response where measurable outcomes do not linearly map to increased exposure. The central hypothesis of this dissertation is that DEHP affects estradiol and testosterone during development that impact social and anxiety behavior and reproductive development in a dose-dependent manner, and that this early exposure has effects several generations later. I evaluated this in two studies. The first used antiandrogenic doses (150mg/kg and 200mg/kg) comparable to a dose that causes transgenerational changes in sperm. DEHP was administered to dams during a gestational window where DNA demethylation and remethlyation and important neurodevelopment occur. The mice that I studied were third generation offspring removed from contact with DEHP. Females from the DEHP-lineage had lower serum corticosterone following restraint stress, and DEHP-lineage males had altered social behavior and reproductive development. The second project was a dose response study with a range of human relevant doses that I hypothesized would be androgenic (5, 40, and 400 μg/kg) administered during gestation and a portion of lactation to imitate human exposure. There were dose and sex-specific outcomes on social and anxiety behavior and reproductive development following direct exposure, as well as increased social behavior several generations later. I also hypothesized that developmental immune deficiency would affect social behavior based on known effects on learning in mice and correlation with behavioral disorders in human. A study using a mouse model of severe combined immunodeficiency (SCID) resulted in impaired social recognition in juveniles that was rescued by transfer of healthy splenocytes. SCID mice also had decreased maternal behavior, which affected social preference in offspring.

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Acknowledgements

I want to give thanks to Erin Harris, Dr. Kimberly Cox, Dr. Paul Bonthuis, Savera Shetty, Aileen Ryalls, and Shatila Zaman who contributed substantially to the completion of the studies in this work. I would also like to thank the other members of the Rissman lab who were friends and educators throughout my graduate school experience: Dr. Jennifer Wolstenholme, Dr. Danielle Stolzenberg, and Dr. Jean Abel. Thank you to my committee: Dr. Iggy Provencio, Dr. Margaret Shupnik, Dr. Patrick Grant and Dr. Michael Scott who have provided guidance and clarity throughout the formation and development of my projects, and the University of Virginia Neuroscience Graduate Program, in particular Tracy Mourton, Nadia Cempre, Dr. Bettina Winckler and Dr. Manoj Patel. Of course, I have to give huge thanks to my mentor Emilie Rissman who has challenged me, encouraged me, and helped me to succeed over the last five years. Lastly, thank you to my wonderful and encouraging personal support system in Charlottesville who have made my time in this town meaningful and a lot of fun; especially Emily Andre, Christine van Hover and Patrick O'Malley. So many other friends and colleagues have also helped to shape my experience here, and I am extremely grateful for all of them.

Dedicated to:

my wonderful family, who I love so much and has never stopped believing in me, especially my mom, Cindy Ranfranz, for always encouraging me to carve out my own path in this world, my dad, Gavin Quinnies, for reminding me how proud he is of my choices and accomplishments, and my sisters and best friends Lauren and Lindsey Quinnies, for keeping me sane and making me laugh each and every day.

Thank you all for being so amazing.

Chapter I

Introduction

i. Social and Anxiety Behavior and Stress response

a. Social Behavior

Genetic and neural regulation:

Social behavior is an important adaptation for mammals in terms of reproduction, survival, maternal care, and community. Multiple peptides, hormones, and genes mediate these behaviors. Particularly vasopressin, oxytocin, serotonin and dopamine (Ebstein, Israel et al. 2010). Additional genes also play roles in certain social behaviors, such as immediate early genes, and thousands of others that are sexually dimorphic and specific to various organisms (Robinson, Fernald et al. 2008).

Hormones have an especially profound effect on the brain during development when sexual differentiation occurs. Proper development of social behavior is guided by a series of organizational androgens and estrogens. Estradiol is produced in the brain and gonads of the developing fetus, and the mother's body (McCarthy 2008). Additionally, it is synthesized from testosterone and has widespread effects on gene expression (**Figure 1**, (Morris, Jordan et al. 2004). Activity of estradiol mediates cell death, synapse morphology and gene expression, having effects on behavior throughout the life of the offspring. Estrogen receptors act as transcription factors, which translocate to the nucleus upon binding. Here, they interact with co-activators to form a complex that allows for interaction with DNA binding sites, or estrogen response elements (ERE), and induce transcription (Reviewed in(McCarthy 2008). Androgen receptors can also act as transcription factors and play an important role in sexual differentiation and behavior (Shang, Myers et al. 2002). Functioning androgen and estrogen receptors are necessary for normal behavior. Both estrogen receptor α knockout mice and male mice with non-functional androgen receptors have impaired social investigation and sex behavior (Wersinger, Sannen et al. 1997, Wersinger and Rissman 2000, Bodo and Rissman 2007, Bodo and Rissman 2008).

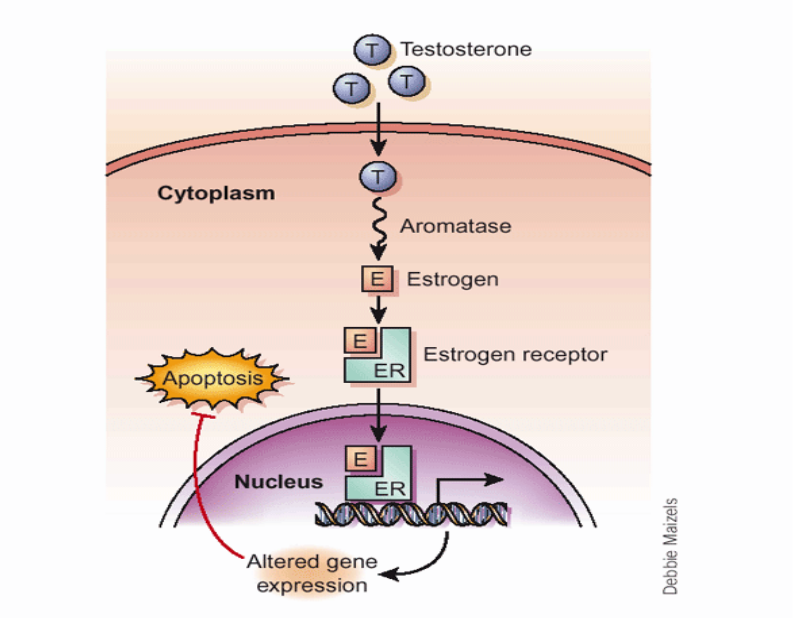

Figure 1. The conversion of testosterone into estrogen.
In male rats, the testes secrete testosterone, which is converted into an estrogen in the brain by the enzyme aromatase. Estrogen then binds to estrogen receptors (ER) to modulate gene expression such that the SDN-POA differentiates in a masculine fashion. Steroid reduces naturally occurring cell death, resulting in more neurons surviving in males than in females. (From Morris, Jordan et al. 2004)

Oxytocin (OT) and vasopressin (AVP) are significant and well-studied regulators of several behaviors, and are modulated by hormone signaling. These two peptides are similar in size and composition and related to one another in terms of location in the brain and behaviors that they control. AVP acts through two receptors, V1a and V1b, while OT has one primary receptor (OTR). Both peptides are produced in magnocellular and parvocellular neurons in the paraventricular and supraoptic nuclei of the hypothalamus, which project to the pituitary gland and other brain regions. AVP is also expressed in the amygdala and bed nucleus of the stria terminalis (BnST) (**Figure 2**, Reviewed in(Benarroch 2013). Male have more AVP cells in the BnST and amygdala and higher AVP fiber density in the lateral septum (Miller, DeVries et al. 1992). Both of these differences are regulated by testosterone-dependent decreases in methylation at the AVP gene promoter (Auger, Coss et al. 2011). Additionally, both AVP and OT are

modulated by estrogen receptor β activity in the hypothalamus of mice and rats (Alves, Lopez et al. 1998, Nomura, McKenna et al. 2002), and OTR synthesis in the amygdala is stimulated by estrogen receptor α (Broad, Curley et al. 2006). Furthermore, hypothalamic OTR density in female rats increases in the presence of circulating estradiol and progesterone (Coirini, Johnson et al. 1992). All of these mechanisms play a part in significant behavioral outcomes. OT promotes maternal care (Pedersen and Prange 1979, Pedersen, Ascher et al. 1982), and the OT receptor and AVP (V1a) receptor in the lateral septum of mice are both positively correlated with nursing and grooming behavior of mouse dams towards pups (Curley, Jensen et al. 2012). Moreover, AVP binding of the V1a receptor is required for social recognition (Bielsky, Hu et al. 2004, Bielsky, Hu et al. 2005), and both AVP and OT in the amygdala are vital for normal social recognition behavior (Ferguson, Aldag et al. 2001, Keverne and Curley 2004, Choleris, Little et al. 2007). V1b knockout mice have impaired social motivation (Wersinger, Kelliher et al. 2004), and the AVP V1b receptor is essential for aggressive attack in mice (Wersinger, Caldwell et al. 2007).

Also of importance for aggressive social behavior is the neurotransmitter serotonin (5-HT) (Garattini, Giacalone et al. 1967, Coccaro, Fanning et al. 2015). Interestingly, serotonin inhibits aggression whereas testosterone facilitates aggression. However, both testosterone and estradiol increase 5-HT receptor expression in the brain of male rats (Nelson and Chiavegatto 2001). In humans, serotonin dysregulation during early development has been implicated in autism (Yang, Tan et al. 2014) and programming of responses to negative social situations (Oberlander 2012). Many studies have hypothesized that the effect of 5-HT on social behavior is due to the fact that higher levels in the brain lead to increased neuroplasticity, which is likely to result in amplified emotional sensitivity to both positive and negative social situations (Kiser, Steemers et al. 2012).

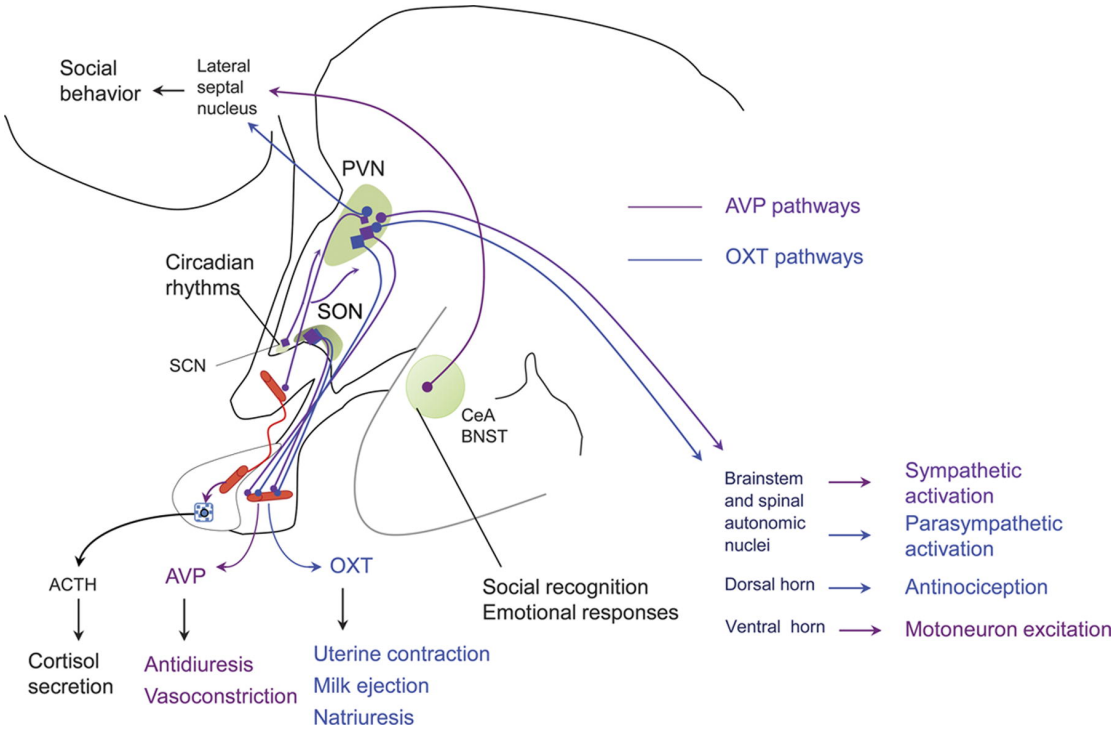

Figure 2: Oxytocin (OXT) and arginine vasopressin (AVP) are synthesized in magnocellular and parvocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei; AVP is also expressed in neurons in the suprachiasmatic nucleus (SCN), central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST),; the extra hypothalamic expression of OXT is in general similar to that of AVP. The axons of magnocellular PVN or SON neurons synthesizing AVP or OXT project to the posterior pituitary, where these peptides are released to circulation. The axons of parvocellular OXT and AVP neurons are also distributed in several areas of the CNS, where these neuropeptides modulate several functions. ACTH = adrenocorticotropic hormone. (Adapted from Bennaroch 2013)

Dopamine is recognized in the social behavior field for its importance to facilitation of partner formation. This neurotransmitter is synthesized in the ventral tegmental area (VTA), and acts via D1-like and D2-like receptors. Dopamine induces the release of OT in rodents and monkeys. It also interacts with OT in pair bonding and maternal behavior, and in conjunction with OTR in the hypothalamus to facilitate male sex behavior (Baskerville and Douglas 2008). In prairie voles, OTR and dopamine (D2) receptors in the nucleus accumbens are both necessary to form pair bonds (Liu and Wang 2003). The mesolimbic dopamine reward pathway is critical for recognition of social reward, as well as drug and food reward (Koob and Le Moal 2001). The major components of this pathway are dopaminergic inputs from the VTA to the nucleus

accumbens, which also receives glutamatergic projections from the prefrontal cortex (Nestler and Carlezon 2006). The prefrontal cortex and nucleus accumbens are essential brain regions for pair bonding as well, highlighting the connection between reward reinforcement and social behavior (Young and Wang 2004). Furthermore, D1 receptor binding in the nucleus accumbens and dopamine interaction with estradiol in the medial preoptic area of the hypothalamus (MPOA) facilitates maternal behavior and sex behavior in rats (Stolzenberg, Zhang et al. 2010, Stolzenberg and Numan 2011).

Other genes play more nuanced roles in social behavior. The initial identification of gene changes in response to social environment was through the study of immediate early genes (IEGs). IEGs are a series of genes that become active shortly after interaction with a stimulus and initiate a cascade of transcription (Clayton 2000). As the technology of sequencing and genome mapping improves, so does the scope of experiments examining gene changes in relationship with behavior. Genetic and epigenetic changes that may not have been predicted are often revealed following analysis this in depth. Some genes are relatively conserved across species and encode evolutionarily relevant transcription factors (Robinson, Fernald et al. 2008). Genes already characterized in different systems are also being found to have additional roles in neuroendocrine behavior circuitry. For instance, CD38, a glycoprotein found on the surface of several immune cells regulates OT secretion. Mice lacking CD38 have decreased social and maternal behavior, and lower levels of circulating OT. These behavioral deficits are rescued by subcutaneous injection of OT, or lentiviral delivery of CD38 in the hypothalamus (Jin, Liu et al. 2007). Discovery of more genes that interact with OT, AVP, dopamine, serotonin and steroid hormone receptors will provide more information about the complex gene networks that contribute to the regulation and modification of social behavior.

In conclusion, social behaviors are diverse and regulated by a complex series of mechanisms controlled by several primary genes and molecules. This extends to maternal behavior, play behavior, sex behavior, and social recognition among others. Social behavior is

important for facilitating many relationships and influenced and controlled by several factors with unique and significant roles.

Environmental influence and social transmission*:*

Environmental factors affect social behavior through steroid hormones and gene transcription. For example, endocrine disrupting chemicals (EDCs) are compounds that interfere with the steroid hormone pathway, and have profound effects on social behaviors and various mechanisms that control these behaviors. EDCs can bind estrogen receptors (Kuiper, Lemmen et al. 1998, Blair, Fang et al. 2000, Takayanagi, Tokunaga et al. 2006, Thomas and Dong 2006, Shanle and Xu 2011) and androgen receptors (Danzo 1997, Kelce, Lambright et al. 1997, Kelce, Gray et al. 1998) affecting gene transcription and social behavior in numerous studies of animal models and humans (Palanza, Howdeshell et al. 2002, Quesada, Fuentes et al. 2002, Veldhoen, Skirrow et al. 2006, Davey, Bodwell et al. 2007, Moral, Wang et al. 2008, Engel, Miodovnik et al. 2010, Swan, Liu et al. 2010, Braun, Kalkbrenner et al. 2011, Wolstenholme, Taylor et al. 2011, Wolstenholme, Edwards et al. 2012, Wolstenholme, Goldsby et al. 2013). Maternal environment and other social interactions also impact social behavior. In humans, negative emotional attention from mothers is associated with antisocial behavior in children (Caspi, Moffitt et al. 2004). In mice, interactions between pups and their mothers during nursing and rearing have lifelong effects on the social behavior of the pups. Low levels of maternal care are repeated in pups with their future offspring, and have been linked to decreased expression of the OT receptor gene (Meaney 2001). The impact of maternal behavior and one specific endocrine disrupting chemical, di-(2-ethylhexyl) phthalate on social behavior will be discussed in more detail in later sections.

b. Anxiety Behavior and Stress response

Overview of anxiety, stress response, and the HPA axis

The hypothalamic-pituitary-adrenal (HPA) axis is an important driver of stress response and anxiety behavior. A series of peripheral and central nervous system responses regulate blood level of glucocorticoids, activity of several brain regions, and hormone release by the pituitary

gland (**Figure 3**). This creates a feedback loop of excitation and inhibition on behavior and chemical output of the brain, pituitary gland, and adrenal cortex of the kidney. Glucocorticoids in the brain and pituitary gland are significant indicators and regulators of stress and anxiety and are synthesized beginning at embryonic day 14.5 in mice (Michelsohn and Anderson 1992) and embryonic day 50 in humans (Goto, Piper Hanley et al. 2006). These hormones are essential for embryonic survival and normal behavioral development and neurodevelopment, particularly in the hypothalamus, amygdala, and hippocampus (Laryea, Muglia et al. 2015), where they bind mineralocorticoid receptors and glucocorticoid receptors to regulate anxiety, stress, and fear responses (Arnett, Kolber et al. 2011). In addition to glucocorticoids, AVP has a significant role in stress and anxiety behavior, and AVP (V1a) receptor knockout mice have reduced anxiety (Bielsky, Hu et al. 2004). AVP and corticotrophin releasing hormone (CRH) are both synthesized and released from parvocellular neurons in the PVN, and together stimulate proopiomelanocortin (POMC) and adrenocorticotropic hormone (ACTH) release from the pituitary (Matthews 2002). In fact, AVP circuitry stimulates ACTH release (**Figure 2**) in rats, mice, and humans (Salata, Jarrett et al. 1988, Mazzocchi, Malendowicz et al. 1997, Benarroch 2013). CRH also initiates 5-HT activity (Nestler, Barrot et al. 2002, Holsboer 2003). CRH injected into the prefrontal cortex increases anxiety behavior in mice by enhancing 5-HT receptor mediated signaling. CRH also amplifies 5-HT signaling in cultured cells by utilizing rapid receptor internalization and recycling to increase surface 5-HT receptor expression (Magalhaes, Holmes et al. 2010).

Not surprisingly, the HPA axis interacts with other steroid hormones in the brain and gonads. Serum testosterone and estradiol are inhibited following stress in both male and female rats (Fenchel, Levkovitz et al. 2015, Lu, Wu et al. 2015). Additionally, testosterone has an anxiolytic effect through androgen receptor (AR) activity. Multiple studies show that female mice and male mice with dysfunctional ARs display increased anxiety behavior in an elevated plus maze (Chen, Brummet et al. 2014). Estradiol also plays a complex role. In female rats circulating estradiol enhances serum corticosterone levels, whereas ovariectomized females lacking estradiol

exhibit a blunted response. Moreover, females that received 120 µg/mL of estradiol subcutaneously had decreased CRH in the PVN following restraint stress (Lunga and Herbert 2004). These studies in combination show that through binding of their receptors, estradiol and testosterone operate in a feedback loop with circulating stress hormones to further regulate the HPA axis, stress response, and anxiety. Because multiple receptors and hormones are involved in these pathways, several different points can be disrupted by chemical and environmental factors. Stress response and anxiety are necessary adaptations for survival, but dysfunction of the mechanisms results in altered behavior and psychiatric disorders.

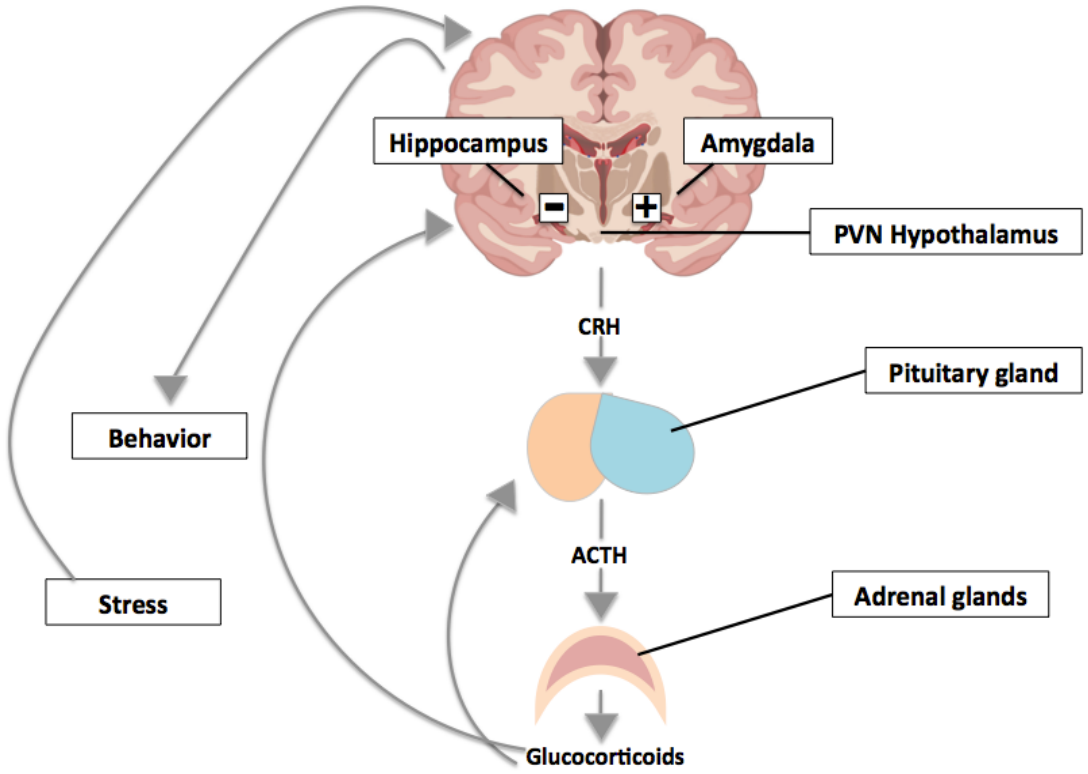

Figure 3: Stress in the environment impacts the hypothalamic-pituitary-adrenal axis. The hippocampus exerts negative feedback on the hypothalamus while the amygdala has an excitatory impact. The paraventricular nucleus of the hypothalamus (PVN) releases corticotrophin releasing hormone (CRH) which binds receptors in the pituitary gland facilitating the release of adrenocorticotropic hormone (ACTH) that binds receptors in the cortex of the adrenal glands that release glucocorticoids. The level of glucocorticoids in the blood and the impact of this circuit on the brain affect behavior.

Environmental influence and social transmission

Environmental factors, prenatal environment, and maternal care have long-term effects on stress and anxiety. In humans, stress during pregnancy and lactation causes an imbalance of stress hormones in the fetus/newborn, and is correlated with altered development of the HPA axis resulting in childhood and adult behavioral disorder diagnosis (Csaszar, Melichercikova et al. 2014). Studies in animals have recreated this and further elucidated the mechanisms behind it. Stress on the mother during pregnancy creates dysresgulation of hippocampal synaptic development, which in turn increases stress response and impairs learning in the offspring (Meaney 2001). When mice undergo restrain stress between days 13-17 of pregnancy, serum corticosterone increases in the stressed dams. The increase is recapitulated in male, but not female fetuses (Montano, Wang et al. 1991). Prenatal stress in mice also causes placental inflammation and hyperactivity in the offspring, which is also male-specific (Bronson and Bale 2014). These sex differences are due to the influence of steroid hormones on HPA response and behavior, and make male fetuses more susceptible to this type of prenatal manipulation. Maternal care after birth has additional long-term effects on stress response (Francis and Meaney 1999), as do other early social interactions.

Chemicals in the environment also affect the stress response and anxiety behavior. Several endocrine disrupting chemicals dysregulate the HPA axis by interfering with steroid hormone signaling. Interestingly, in female mice exposed to estrogenic chemicals bisphenol-A and ethinyl estradiol at human relevant doses, behavior in a spatial task and anxiety test was masculinized in females exposed to ethinyl estradiol and anxiety behavior was increased in females exposed to BPA (Ryan and Vandenbergh 2006). Several other studies report increase in anxiety following BPA exposure as well (Patisaul, Sullivan et al. 2012, Xu, Hong et al. 2012, Diaz Weinstein, Villafane et al. 2013). Phthalates and other endocrine disruptors also affect stress and anxiety by interacting with enzymes and receptors along the steroidogenisis pathway (Ryan and Vandenbergh 2006, Carbone, Ponzo et al. 2013, Lee, Chiang et al. 2015, Park, Cheong et al.

2015). The impact of one specific phthalate, di-(2-ethylhexyl) phthalate, on stress response and anxiety behavior will be further explored in subsequent sections and chapters.

ii. The immune system and behavior

The immune system has been linked to changes in behavior in humans and plays a role in learning and memory behavior. Previous studies using Severe Combined Immunodeficiency (SCID) mice showed that adult males have impaired spatial learning, which is rescued with splenocyte, or purified T-cell, replacement facilitated by T-cell mediated release of anti-inflammatory cytokine Interleukin-4 in the meninges of the brain (Brynskikh, Warren et al. 2008, Derecki, Cardani et al. 2010). SCID mice lack adaptive immunity due to a spontaneous mutation in the *Prkdc* gene on chromosome 16, which impairs the recombination of antigen receptor genes and results in the interrupted development of B and T cells (Bosma, Custer et al. 1983, Bosma and Carroll 1991, Hsiao, McBride et al. 2012).

Adult mice lacking the recombination-activating gene RAG1 are deficient in social recognition tasks (McGowan, Hope et al. 2011). RAG1 (along with RAG2) mediates variable (diversity) joining recombination, necessary for the maturation of B and T cells (McGowan, Hope et al. 2011). This variable joining recombination is the same process that is disrupted by the *Prkdc* gene in SCID mice, causing their lack of T and B cells (Bosma, Custer et al. 1983, Bosma and Carroll 1991). Additionally, some mouse models for autism exhibit social behavior deficits and have deficiencies in immune function. For example, there is evidence for immune system disruption in the ASD mouse model BTBR *T+tf*/J (BTBR). The BTBR strain has higher levels of serum IgG and IgE, as well as elevated expression of pro-inflammatory cytokines IL-33, IL18, and IL1β in the brain compared to B6 control mice (Heo, Zhang et al. 2011). Furthermore, previous studies show decreased social behavior of offspring whose dams had infections during gestation, which is likely linked to increased cytokine activity (Malkova, Yu et al. 2012).

Additionally, reversal of social behavior deficits and rescue of a proinflammatory phenotype in a maternal immune activation (MIA) model of poly (I:C) injection, are noted after MIA offspring received bone marrow transplantation from immunologically healthy mice (Hsiao, McBride et al. 2012). Importantly, immune system activity also has the potential to affect maternal behavior (Hood, Dreschel et al. 2003, Meyer, Schwendener et al. 2006). This can change the behavior of progeny (Weaver, Cervoni et al. 2004) and is often overlooked in studies with disrupted maternal immune status.

Alterations in immune function affect stress and anxiety behavior as well. In mice, stress during early pregnancy stimulates placental expression of proinflamatory cytokine genes IL-6 and IL-1β, prefrontal cortex expression of D1 dopamine receptors, and stress related hyperactivity specifically in males. This phenotype is rescued by administration of antinflammatory drugs to the mother (Bronson and Bale 2014). Maternal immune activation and stress induced increase of corticosterone and proinflammatory cytokine TNFα in a mouse model of multiple sclerosis also results in increased anxiety behavior in males (Majidi-Zolbanin, Doosti et al. 2015). Early prenatal exposure to lipopolysaccharide also results in increased anxiety behavior and reduced serotonin in the hippocampus (Depino 2015).

Immune status is linked to several psychiatric disorders in humans (Michel, Schmidt et al. 2012). Specifically, autism spectrum disorder (ASD) has been correlated with infection during pregnancy (Atladottir, Thorsen et al. 2010, Zerbo, Qian et al. 2013), and with familial autoimmune disease (Comi, Zimmerman et al. 1999, Atladottir, Pedersen et al. 2009, LY and Mostafa 2014). Although cognitive ability has been examined in mice that undergo developmental immune dysfunction, and social and anxiety behavior have been evaluated following acute immune challenges, few studies have been conducted on social behavior and developmental immune dysfunction.

iii. Endocrine Disruptors

a. History:

The idea has existed for decades that chemicals released into the environment are able to mimic and alter normal hormonal development (McLachlan and Newbold 1987). Chemicals in the environment that interfere with hormone pathways in wildlife populations and humans do indeed exist, and are considered endocrine disrupting chemicals (EDCs). There are thousands of compounds that meet these criteria and they often have adverse effects. In fact, EDCs have been contaminating the environment since the 1930s and affecting reproductive development and neurodevelopment in humans and animals, often causing effects that persist through generations (Colborn, vom Saal et al. 1993). Humans and animals come into contact with EDCs on a regular basis through food packaging, plastic toys, receipts, medical equipment, textiles, household dust, workplace environments, fish, meat, and drinking water (Frye, Bo et al. 2012). As research provides information that restricts the use of these chemicals, new EDCs continue to be manufactured (Kinch, Ibhazehiebo et al. 2015, Lioy, Hauser et al. 2015). Several types of EDCS exist such as bisphenol-A (BPA), phthalates, polychlorinated biphenyls (PCBs), flame-retardants, dioxins, vinclozolin, as well as other phytoestrogens, pesticides, and heavy metals that interfere with hormones. Furthermore, even EDCs that have been banned, such as PCBs, still remain traceable in the infrastructure of the environment and in human blood samples (Pan, Daniels et al. 2010, Marek, Thorne et al. 2013).

b. Effects on reproduction, neurodevelopment, and behavior

Hormone activity is a crucial element of development, reproduction and normal behavior in adolescence and adulthood. Due to diverse effects on steroid hormone receptors, different EDCs impact important processes in various ways. This is particularly important to consider during early development. During gestation and early life, hormones play a prominent role in the formation of several brain areas and shaping differences between males and females. These early

changes can have long lasting effects, some of which are heritable in future generations removed from exposure.

As the name suggests, a popular avenue for studying the effects of EDCs is the reproductive system. Not surprisingly, EDCs do alter normal reproductive development in humans and many animal models. Several EDCs, such as BPA, PCBS, phthalates and vinclozolin, modulate circulating testosterone and estradiol during developmental exposure, resulting in malformation of male and female reproductive tracts (Colborn, vom Saal et al. 1993, Cooper and Kavlock 1997). Importantly, EDCs also have effects on neurodevelopment and behavior sometimes causing multigenerational and transgenerational changes (Anway, Cupp et al. 2005, Anway and Skinner 2006). Because of the extensive effect of EDCs on steroid hormones, certain effects are specific to males or females, and some sex differences shift across generations (Kubo, Arai et al. 2003, Gioiosa, Fissore et al. 2007, Wolstenholme, Edwards et al. 2012). A well-studied example of this is social behavior in juvenile mice exposed to Bisphenol-A (BPA). Exposure to BPA lowers gene transcripts of essential social neuropeptides oxytocin and vasopressin in mouse embryo brains, and also alters social behavior in juveniles. Interestingly, side-by-side interactions and social investigation are decreased in the BPA exposed mice from the second (F2) generation, but increased in the fourth (F4) generation. This is likely because of modification to estrogen receptors in the animals that were exposed to BPA as germ cells that did not take place in the F4 mice (Wolstenholme, Edwards et al. 2012).

Often, EDCs are studied in only males or females, depending on the sex thought to suffer from toxicity based on the known and suspected hormonal mechanisms of the chemical in question. For example, phthalates are commonly considered “antiandrogenic,” meaning lowering testosterone, so males are traditionally studied. However, these effects on testosterone are dose dependent, expanding the need for additional research.

c. Dose-response studies:

Toxicity of endocrine disruptors is commonly assessed by administering a range of high doses to animals, but EDCs often have non-linear non-monotonic response curves (Welshons, Thayer et al. 2003) and can cause changes at low and intermediate doses (**Figure 4**). In recent years, scientists have been more diligent in researching a wide range of doses and taking low doses into account. Historically, it has been thought that higher doses mean more pronounced effects, and therefore only high doses were assessed to determine the lowest observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL). This has been challenged by many studies in animals and humans of several different EDCs (Vandenberg, Colborn et al. 2012).

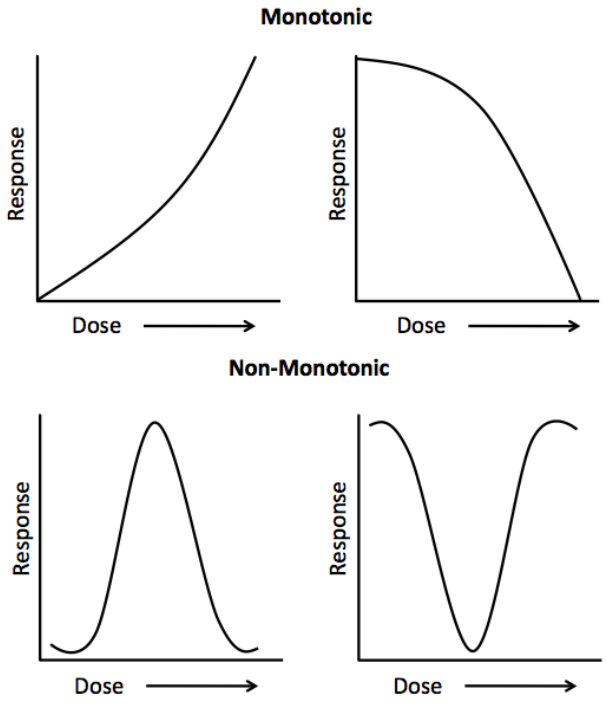

Figure 4: A traditional, monotonic dose curve is represented by a linear response to dose administration of chemicals. However, many EDCs have a non-monotonic dose response where steroid hormone levels or gene expression respond differently to varied doses in a non-linear way.

In order to best understand the extent of effects for a chemical, several doses, time frames, and routes of exposure must be considered. Toxicologists, other scientists, and the Environmental Protection Agency have published comprehensive reports detailing the important factors to take into consideration when conducting and evaluating dose response studies (Vandenberg, Colborn et al. 2012). Since endogenous hormones act at extremely low levels, EDCs must also be evaluated at low levels. Certain EDCs are actually released into the environment at high levels, so it is important to study doses that correspond to all levels of intake. Essentially, a full range of doses is necessary to accurately predict the full range of effects on humans.

d. Transgenerational effects

Human studies of endocrine disruptors show what actual effects on the population are, but do not permit researchers to completely separate direct effects from heritable changes or remove confounding factors. Animal studies offer insight into this by allowing research of direct results of exposure as well as the study of animals generations removed from exposure. Transgenerational effects are changes in biology or behavior transmitted from one generation to another, and are usually studied following exposure to an event or substance. For something to be truly transgenerational, it has to be observed several generations after exposure to the factor in question. If a pregnant animal (or human) is exposed to a chemical, the mother, the fetus, and the germ cells of the fetus all come into contact. Therefore, the generation after the germ cells is what must be studied in order to observe truly heritable changes (**Figure 5**). A number of EDCs have transgenerational effects on gene expression, behavior, and physiological development (Anway, Cupp et al. 2005, Skinner and Anway 2005, Anway and Skinner 2006, Stouder and Paoloni-Giacobino 2010, Doyle, Bowman et al. 2013, Wolstenholme, Goldsby et al. 2013, Kawano, Qin et al. 2014, Zhang, Zhang et al. 2014). The mechanisms driving these stable changes is not always clear, but is made more evident by separating direct effects from transgenerational effects.

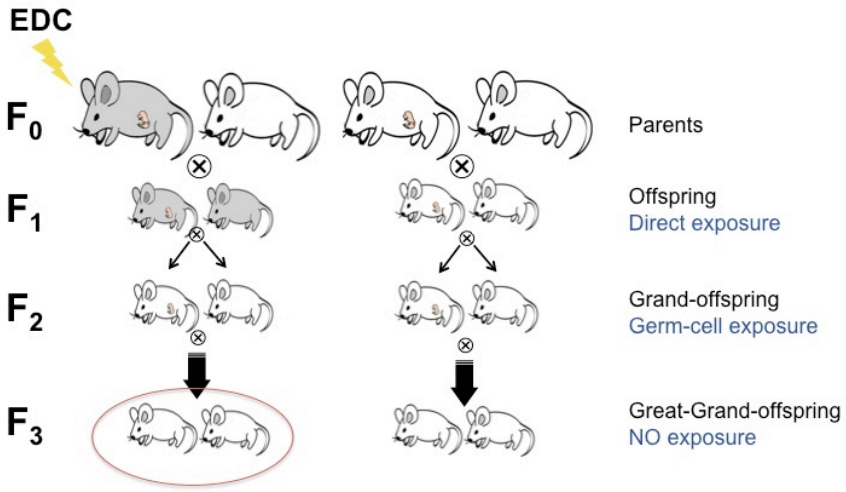

Figure 5: Transgenerational studies involve animals completely removed from contact with the chemical being tested. When the F0 dams receive a dose the fetus and the germ cells of the fetus (eventual F2 generation) are exposed as well.

iv. DEHP

a. Overview

Phthalates are a common EDC, and one of the most prevalent of these compounds is di(2-ethylhexyl) phthalate (DEHP) with its yearly production in the US estimated at 1-4 million tons and increasing (McKee, Butala et al. 2004, Halden 2010). It is used in flexible plastic production, specifically that of polyvinyl chloride (PVC) and is present in food packaging, medical equipment, and synthetic flooring. DEHP leaches easily from PVC as it does not form a covalent bond (Latini, Verrotti et al. 2004). Daily human intake levels have been estimated between 6 and 21 μg/kg/day, but are variable based on age and environment with children having higher measurable levels (Crinnion 2010). Furthermore, DEHP can be absorbed easily through the skin (Hopf, Berthet et al. 2014) and crosses the placenta (Singh, Lawrence et al. 1975). There are DEHP metabolites in breast milk, amniotic fluid, and cord blood making contact with fetuses

a regular occurrence (Latini, De Felice et al. 2003, Silva, Reidy et al. 2004, Fromme, Gruber et al. 2011). Infants receiving medical treatment can be exposed to up to 100 times as much DEHP as adults (Koch, Preuss et al. 2006).

Studies in humans and animals are beginning to shed more light on the variety of consequences of exposure. To date, DEHP is linked to reproductive system disorders, altered neurodevelopment, anxiety and social behavior, and obesity and disease risk in both animal models and humans (Swan, Liu et al. 2010, Testa, Nuti et al. 2012, Carbone, Ponzo et al. 2013, Tseng, Yang et al. 2013). The following sections will evaluate what is already known and what is lacking about how DEHP acts physiologically and the behavioral, neurological, reproductive, and metabolic impact on the human population and environment.

b. Effects on the steroid hormone pathway and mechanism of action

Existing research clearly shows that DEHP affects steroidogenesis at multiple points. Specifically, exposure to DEHP *in utero* and during lactation at doses that are comparable to average human levels lowers serum testosterone and aldosterone in rats and mice (Do, Stahlhut et al. 2012, Martinez-Arguelles, Campioli et al. 2014). Higher doses within and beyond calculated average human intake elevate maternal and fetal testosterone, and extremely high doses out of the range of normal daily human intake lower maternal and fetal serum testosterone (Do, Stahlhut et al. 2012). This U-shaped non-monotonic dose curve shows a range of non-linear steroid hormone response at different doses, meaning that the highest doses do not necessarily have the most robust effects. A primary metabolite of DEHP, mono-(di-ethylhexyl) phthalate (MEHP), is a necessary component for these changes in testosterone levels *in vitro* and *in vivo*, but *in vitro* studies indicate that MEHP is not a competitive ligand for the androgen receptor (AR) (Parks, Ostby et al. 2000). Therefore, the changes in testosterone must occur via other pathways or mechanisms, and not direct AR binding.

One possibility is that the effect on testosterone is mediated by estrogen receptor activity. In a competitive binding assay with human ERα, DEHP binds to the receptor, but only at concentrations between 100μM and 100mM indicating that DEHP has a binding capacity 1000 fold lower than estradiol. These are supraphysiological conditions, and comparable levels have never been measured in humans (Ohashi, Kotera et al. 2005). Another study using high dose DEHP treatment in a human breast cancer cell line elicits enhanced cell activity and proliferation similar to those exhibited in the presence of estradiol, but that study did not produce any additional evidence of direct binding to estrogen receptors (Jin 2008). This information suggests that ER activity may play a role in animal and cell culture studies using extremely high doses, but that this is an unlikely candidate as a contributing mechanism in physiologically relevant doses. There are *in vivo* high dose effects of DEHP on ERα gene expression (*Esr1*) in the ovaries of mice that are mediated by peroxisome proliferator-activated receptor alpha (PPARα). In mice receiving 80 mg/kg of DEHP beginning at 12 weeks of age and continuing through the F2 generation *Esr1* expression decreased, especially in F1 and F2 mice. However, this gene expression change did not occur in PPARα knockout mice (Kawano, Qin et al. 2014) suggesting that PPARα binding is necessary for DEHP effects on *Esr1* expression.

Importantly, additional studies also show that DEHP acts through the PPAR signaling pathway, using its metabolite MEHP is a ligand (Feige, Gelman et al. 2007). MEHP activates two of the three PPAR isoforms: PPARα and PPARγ (Maloney and Waxman 1999, Benjamin, Flotho et al.). The PPAR pathway is important for proper neural development (Borch, Metzdorff et al. 2006, Heneka and Landreth 2007, Gonzalez, Corton et al. 2009) and directly affects steroid hormone production via cholesterol, Cyp17, and 3βHSD, Cyp19 (aromatase), these hormones in turn affect androgen and estrogen metabolism and production of estrogens, progesterone and testosterone (Borch, Metzdorff et al. 2006). Specifically, PPARγ ligands at endogenous levels inhibit aromatase, the enzyme that converts testosterone to estradiol (Lovekamp and Davis 2001).

Therefore, this ligand activity may be responsible for the effects on testosterone observed in animal studies. In fact, DEHP does disrupt aromatase activity in an age and sex dependent manner in rats (Andrade, Grande et al. 2006). This may also explain some estrogenic effects, as disruption of aromatase driven conversion of testosterone to estradiol would change circulating levels of both of these hormones. In fact, activity of PPARs can have a wide range of consequences. In addition to metabolizing ligands of PPARs, exposure to DEHP *in vitro* actually increases expression of PPARγ, and through the activation of PPARγ in cultured neurons, increases the expression of caspase-3 activating protein Trim17, which induces cell death (Lin, Chen et al. 2011). Furthermore, MEHP binding to PPARα and this binding is thought to be at least partially responsible for adverse biological reactions to DEHP in the liver and male reproductive tract where this receptor is abundant (Lapinskas, Brown et al. 2005).

DEHP also activates Retinoid X receptors, and actually has a stronger binding affinity for Retinoid X Receptors (RXRS) than PPARs, which may compound the induction of cell death (Sarath Josh, Pradeep et al. 2013). Although this relationship is not well characterized, RXRs are crucial for normal development, and RXR knockout mice are embryonic lethal (Wendling, Chambon et al. 1999). RXRs also have a heterodimeric partner, constitutive androstane receptor (CAR), a nuclear receptor important for the regulation of hepatic enzymes and metabolism. DEHP can activate CAR in vivo, observed by gene expression in livers of mice exposed to 20 mg/kg of DEHP for 3 weeks, and is also seen at comparable doses in hepatic cell lines (Eveillard, Mselli-Lakhal et al. 2009). This suggests the importance of heterodimers and interactive receptor pathways in the complex activity of DEHP and MEHP. DEHP also disrupts the MAPK and the NF-κβ pathway. In female mice given 1000mg/kg of DEHP/day, immunocytochemistry shows a down regulation of phosphorylated ERK and NF-κβ staining in the endometrium (Li, Yu et al. 2012). Although disruption of these pathways could have significant and widespread effects,

these doses are not biologically relevant and are likely more indicative of decreased fetal viability in contact with large quantities of DEHP.

DEHP metabolite MEHP binds and significantly inhibits 11β-HSD2, the enzyme that inactivates corticosterone, in murine gonadotrope cells in a dose-dependent manner, converting corticosterone to inactive 11-deoxycorticosterone (Hong, Li et al. 2009, Zhao, Chu et al. 2010). Although downstream effects of this activity are not yet well studied, it is thought that DEHP causes increased corticosterone by inhibiting the activity and expression of 11β-HSD2. This inactivation is essential for preventing over activation of the HPA axis in the fetal environment. Thus disruption of this enzyme and increases in corticosterone during this time are likely to have long term effects on HPA axis development and activity.

Additional research has identified gene changes that coincide with hormone changes, which provide more insight into potential mechanisms. In DEHP adult male rats that were exposed to 1 mg/kg during gestational days 14-birth, hormone-sensitive lipase and phosphoenolpyruvate carboxykinase 1 (*Pck1*) in adrenal glands correlates with lower serum aldosterone (Martinez-Arguelles, Campioli et al. 2014). These changes could be the result of DEHP and its metabolites on other enzymes in the steroid hormone pathway via PPARs, 11β-HSD2 or other unknown receptors. Another explanation is that DEHP interferes with these pathways through the immune system. DEHP activates the Ca/CaN/NFAT signaling pathway *in vitro*, which has a major role in T-cell activation and function. This pathway affects cytokine production that causes inflammation and active immune response, and has multiple downstream targets, including interleukin-4, a cytokine with anti and pro-inflammatory properties (Pei, Duan et al. 2014). Depending on the dose, DEHP both positively and negatively modulates IL-4 production in mouse splenic lymphocytes (Tonk, Verhoef et al. 2012, Pei, Duan et al. 2014). Effects on regulation of other cytokines are conflicting in different reports (Butala, David et al. 2004, Lee, Park et al. 2004, Larsen and Nielsen 2007).

DEHP and its metabolites have a wide range of actions, several of which still have not been thoroughly examined or researched. Various systems are affected by the presence of this compound. More *in vivo* and *in vitro* studies with biologically relevant doses are necessary to further assess the mechanism of action of DEHP and its metabolites. Present research from several labs signifies that DEHP disrupts many points on the steroidogenesis pathway and has differing effects depending on dosage, cell type, window of administration, avenue of administration, animal model, and sex (**Figure 6**, reviewed in(Hannon and Flaws 2015). Information is rapidly expanding regarding which receptors can be bound by DEHP and its metabolites and how hormone levels change in regard to dosage. Future analysis will indicate more specific results for different cell types.

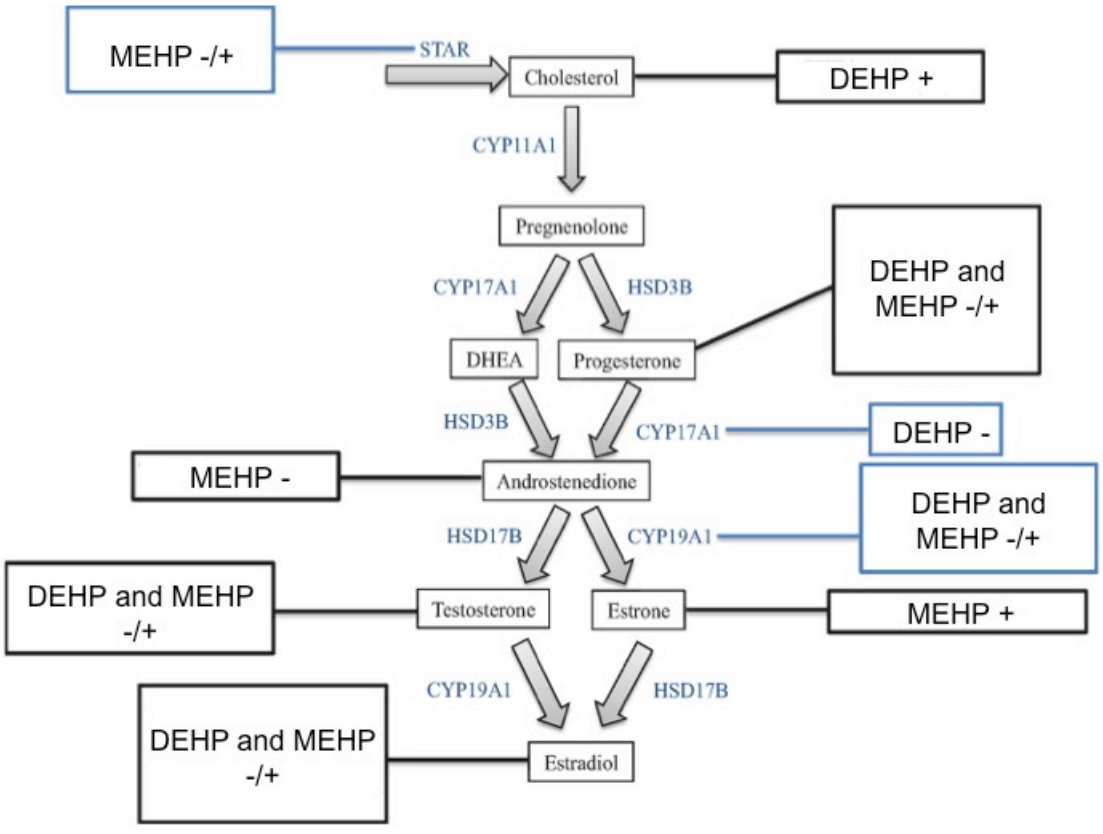

Figure 6. Phthalates alter steroidogenesis. This figure is a summation of the major findings on the effects of phthalates on steroidogenesis. Black text boxes connected to hormones outline the major effects of phthalates on the levels of that hormone. Blue text boxes connected to steroidogenic enzymes outline the major effects of phthalates on the mRNA and/or protein levels of that enzyme. (Adapted from Hannon and Flaws 2015)

c. Effects on reproductive function

Effects of DEHP on the reproductive system are due to disruption of the steroidogenesis pathway, leading to changes in levels of androgens and estrogens as well as adrenal glucocorticoids (Hong, Li et al. 2009, Do, Stahlhut et al. 2012). As mentioned in the previous section, changes in testosterone are the most well studied hormonal effects of DEHP exposure. A 250 uM dose of DEHP inhibits testosterone synthesis *in vitro* in immature and adult rat testicular Leydig cells (Svechnikov, Svechnikova et al. 2008). Comparable doses inhibit testosterone *in vivo*. However, male mice exposed prenatally to lower (physiologically relevant) doses have increased serum testosterone and corresponding effects on anogenital distances (where reduced anogenital distances are indicative of lower fetal testosterone levels) (Do, Stahlhut et al. 2012). In humans, DEHP metabolites are positively correlated with decreased sperm count, lower testosterone levels (Specht, Toft et al. 2014), disrupted sexual differentiation, and dysregulated reproductive development (Gray, Ostby et al. 2000, Moore, Rudy et al. 2001). Data on anogenital distance in human samples is correlated with DEHP exposure, and indicates reduced anogenital distance with increased metabolite levels (Swan, Main et al. 2005).

Further research in female mice shows that DEHP affects implantation and mRNA expression of estrogen receptor α, progesterone receptor and E-cadherin in the endometrium at a variety of doses (Li, Yu et al. 2012). Further estrogenic effects have been shown in adult female rats treated with 1000mg/kg DEHP as adults where estradiol levels in serum are lower than control animals during proestrus, yet higher than controls during estrus (Lovekamp-Swan and Davis 2003). *In vitro*, cultured ovarian granulosa cells show suppressed estradiol production in the presence of MEHP (Liu and Hsueh 1986). DEHP also elevates progesterone levels in the blood of sheep, and in turn lowers pulsatile luteinizing hormone secretion significantly below controls. This results in deficient corpora lutea, possibly due in part to dysfunctional hypothalamic feedback (Herreros, Encinas et al. 2013). In addition to the developmental issues mentioned previously, these acute effects are significant, especially for adult females that may

become pregnant. This is particularly relevant because DEHP levels in human cord blood have been correlated with decreased birth size, specifically in female infants (Huang, Li et al. 2014), and preterm birth (Ferguson, McElrath et al. 2014).

Importantly, some of these changes are likely to have heritable outcomes. Transgenerational changes have been observed in the testes of mice exposed to DEHP. F3 mice, two generations removed from DEHP exposure, have a loss of germ cell organization and abnormal cell morphology in the epididymis as well as lowered sperm cell count and reduced sperm motility (Doyle, Bowman et al. 2013). These heritable effects are expected to be the result of epigenetic mechanisms. There are a few good candidates for the mechanism behind this result. Perhaps the most noteworthy is the 10% increase in global DNA methylation and DNA methyltransferase expression in the testes of male mice exposed to DEHP only *in utero* (Wu, Zhu et al. 2010). Another interesting possibility is the influence of imprinted genes. Oocytes of fetal mice exposed to physiologically relevant levels of DEHP *in utero* and their F2 offspring have a lower percentage of CPG sites methylated in the maternally imprinted *Igf2r* and the paternally imprinted *Peg3* differentially at the methylated regions (DMRs) (Li, Zhang et al. 2014).

Together, these results indicate that DEHP has many effects on the reproductive systems of both males and females as a result of direct exposure during adulthood, contact during development, and heritable effects in generations following but entirely removed from contact. The outcomes of high exposure levels differ from lower ones and the dose most relevant to mimic human exposure in animal studies is somewhat unclear. Furthermore, human populations are more representative of cumulative effects of DEHP exposure across generations than isolated effects of DEHP *in utero*. Another major issue with these studies is that the majority of them are conducted using doses outside of the typical human range of exposure. Future research needs to compare human studies to animal studies at biologically relevant doses, and further research in the brain needs to be done to better characterize which effects are the result of direct impact on the ovaries and testes and what is due to hypothalamic and pituitary feedback.

d. Effects on neurodevelopment and the brain

Perhaps the most comprehensive *in vivo* neuroscience finding to date comes from a study done in the rat hypothalamus showing that *in utero* exposure to DEHP affects neonatal and juvenile aromatase in the hypothalamus in a non-monotonic dose dependent manner. In this study, postnatal day 1 (P1) male rats show increased levels of aromatase when exposed to high doses and decreased aromatase levels at lower doses more comparable to human exposure levels. On the other hand, female rats exhibit increased aromatase levels in response to DEHP exposure during gestation on P22, but not on P1, indicating a sex and age sensitive response to DEHP (Andrade, Grande et al. 2006). Exactly how DEHP is affecting aromatase is not certain, but PPAR ligands inhibit aromatase (Lovekamp and Davis 2001), and MEHP binding to PPARS as an agonist, antagonist, or partial agonist is considered to be the reason DEHP exposure affects this step in the hormone pathway.

DEHP is also anticipated to influence neurodevelopment by affecting cell death and proliferation. As previously mentioned, DEHP activates Trim17 in neuronal culture, which in turn induces cell death via caspase 3 activation. This is also mediated through PPARs, specifically PPARγ (Lin, Chen et al. 2011). DEHP has neurotoxic effects in *C. elegans* as well, which is linked to oxidative stress (Tseng, Yang et al. 2013). Further evidence of cell death caused by DEHP in mice has been seen in the form of a decrease in whole brain weight. Following exposure to DEHP at a dose of 1mg/kg during gestational days 8-17, lower brain weights in 6-week-old mice and lower overall body weights in 2-week-old mice were observed (Tanida, Warita et al. 2009). Moreover, a 30mg/kg dose of DEHP administered *in utero* and during lactation increases GABA in the hypothalamus of male rats (Carbone, Szwarcfarb et al. 2010). GABAergic activity has effects on multiple behaviors, and the hypothalamus is a highly connected brain region. There are also effects of DEHP in the hippocampus. In mice, a high 200mg/kg dose delivered during mid gestation and throughout lactation down regulates the expression of ERβ in the hippocampus of adults and pubertal females, and AR in pubertal males.

This same study showed inhibited phosphorylation of ERK 1 and 2 in the hippocampus during puberty for both sexes (Xu, Yang et al. 2014). Although much more research remains to be done in this area, general effects on various pathways, multiple brain regions, and behavioral results demonstrate a vast and significant effect of DEHP on the brain.

e. Behavioral changes

Current data on DEHP shows that DEHP alters behavior in animal models, as well as in humans. Levels of DEHP and its metabolites in urine samples have been correlated with ASD diagnosis in children (Testa, Nuti et al. 2012), reported symptoms of ADHD in school-age children (Kim, Cho et al. 2009), and risk of attention deficit disorder (Chopra, Harley et al. 2014). Further human data shows prenatal exposure to DEHP is associated with reduced masculine play in preschool aged males (Swan, Liu et al. 2010). In infants, males and females show different patterns of behaviors in response to maternal phthalate concentration in urine during pregnancy. Female alertness and motor performance is decreased in infants exposed to higher levels of phthalates *in utero*. In contrast, males showed a possible increase in motor ability with increasing concentrations of phthalates in maternal urine (Engel, Zhu et al. 2009).

Although human behavioral studies are important, these results are complicated, and can be difficult to interpret on their own, which is why animal behavior research is so crucial. Studies in animals have important implications for understanding human outcomes of DEHP exposure, as the administered dose can be effectively controlled in a laboratory setting. Because of the well studied effects on steroid hormone pathways, the animal work to date focuses primarily on anxiety and sex behavior. Data from rats tested on the elevated plus maze show that postnatal exposure of 30mg/kg of DEHP to a lactating dam on postnatal days 1 through weaning causes anxiety-like behavior in male offspring, but not females. Additionally, males exposed to DEHP have lower levels of testosterone than controls, and that the anxiety phenotype is rescued in adults with peripheral testosterone injection (Carbone, Ponzo et al. 2013). When DEHP is given *in utero* and throughout weaning at a high dose of 200 mg/kg, there are sex-specific effects on anxiety

behavior in adults. In this study, females spend less time in the open arms of the elevated plus maze (Xu, Yang et al. 2014). There are direct effects of DEHP metabolites on glucocorticoids (Hong, Li et al. 2009), which indicate that this is likely to be an acute effect of hormones.

The effects on sex behavior have been mainly observed in males due to the categorization of DEHP as antiandrogenic in high doses. Male rats exposed to 375 and 1500 mg/kg of DEHP (but not 750mg/kg) during gestation and lactation have reduced mounting behavior and failure to ejaculate (Moore, Rudy et al. 2001). An additional study in rats also found no changes in male mounting behavior at the 750 mg/kg dose in offspring following administration to dams on gestational day 14 to postnatal day 3 (Gray, Ostby et al. 2000), suggesting that exposure early in gestation may be essential for the development of this behavior. This is surprising given that actions of endogenous testosterone and masculinization of the brain that are associated with sex behavior occur late in gestation (Arnold and Breedlove 1985). Thus, more detailed research on timelines of dosage is necessary to properly interpret these findings.

This information highlights the need for further behavioral research, especially that on social and sexually dimorphic behavior. To date, only one other animal study has been done looking at the effects of DEHP on social behavior. This study found that administration of 30mg/kg of DEHP to dams during gestation resulted in decreased social interaction in the offspring (Lee, Chiang et al. 2015). While this research is important, it only reports on one dose, and does not differentiate between the effects on males and females. DEHP clearly affects sex behavior and anxiety behavior in animal studies, which is to be expected given the interference with steroid hormone pathways. With continued animal research on a diverse behaviors conducted using a wider range of biologically relevant doses, we will begin to better understand the human data and the important implications that it has.

f. Obesity and disease risk

As DEHP exposure has been shown to have a variety of effects on different systems and pathways, it is thought to be involved in several different metabolic issues. Most exemplary,

DEHP is involved in mechanisms related to obesity, particularly through the interaction with PPARs (Desvergne, Feige et al. 2009). White adipose tissue development is dependent on PPARγ, an established target of DEHP metabolite MEHP as mentioned previously, and MEHP induces adipogenesis via this PPAR receptor (Feige, Gelman et al. 2007). DEHP has been shown to have strong effects in PPARγ activation, but may also be increasing obesity by observed anti-androgenic effects, or potential changes in thyroid activity (although thyroid results are inconsistent) (Hatch, Nelson et al. 2010). This is further clarified by the similar binding effects and agonist properties of MEHP to Rosiglitazone, a pharmaceutical PPARγ agonist (Desvergne, Feige et al. 2009). Furthermore, DEHP impairs gene expression of glucose transporters and insulin receptors *in vitro* (Rajesh and Balasubramanian 2013). Lactational exposure to DEHP impairs insulin signaling and glucose oxidation in rats (Herreros, Encinas et al. 2013). These changes following exposure may also contribute to risk for type 2 diabetes.

DEHP exposure has also been hypothesized to increase cancer risk. DEHP has carcinogenic outcomes *in vitro*. In a human gastric carcinoma cell line, MEHP causes a variety of mutations and DNA damage (Lin, Wu et al. 2013). Some of these effects have also been recapitulated *in vivo*. In mice, exposure to DEHP induced liver tumors, but PPARα knockout mice did not develop these tumors, indicating that PPARα is a likely mechanism for interference in the liver. However, DEHP-induced toxicity in testis, kidney, fetus and embryo were still observed in PPARα null-mice, showing the importance of different receptors in different systems (Peters, Cattley et al. 1997). Moreover, in a two year carcinogenic assay with exposure of 139, 845, and 3147 mg/kg/day of DEHP, mice were subject to increased incidence of liver cancer and testicular and renal degeneration (Wood, Jokinen et al. 2014). Liver abnormalities were also seen in juvenile rhesus monkeys that received plasma transfusions from DEHP-containing PVC blood bags (Kevy and Jacobson 1982).

In addition to the DEHP mediated activation of cell death pathways in neuronal culture (Lin, Chen et al. 2011), cells in a human gastric epithelial cell line exposed to DEHP were less

viable and had higher caspase-3 activation (Lin, Wu et al. 2013). DEHP also has a general effect on the immune system. It alters the Th1/Th2 balance in cultured splenic lymphocytes, potentially by modulating cytokines (Pei, Duan et al. 2014), and Th2 cytokine production is reduced in the serum of mice exposed to DEHP *in utero* (Shin, Lee et al. 2014). Furthermore, In mice, low dose oral exposure to DEHP increases mast cell degranulation and pro-inflammatory cytokine expression (Sadakane, Ichinose et al. 2014). In humans, DEHP has been linked with dermatitis in children (Choi, Kwon et al. 2014, Wang, Lin et al. 2014). Similar effects are seen in mouse models (Sadakane, Ichinose et al. 2014), which may indicate allergy susceptibility and general immune system dysregulation.

v. Conclusions and summary

Social and anxiety behaviors are necessary adaptations for humans and animals, which are influenced by a diverse set of components. Changes in these behaviors are often driven by environmental factors. In order to better understand the outcomes, we must carefully study each component. I have outlined in this introduction the known relevance of EDCs and the immune system on social and anxiety behavior, and the impact of EDCs on neurodevelopment and reproductive development as well as the areas that need to be researched more thoroughly.

The immune system has known effects on cognition, and is linked to social and behavioral disorders. Immune deficient mice have impaired learning (Brynskikh, Warren et al. 2008, Derecki, Cardani et al. 2010), and some animal models of behavioral disorders also have dysregulated immune function (Heo, Zhang et al. 2011). To some degree, immune influence on social behavior has been studied in animals, but major elements remain unclear. Current animal studies focus primarily on acute immune activation (Malkova, Yu et al. 2012, Aavani, Rana et al. 2015, Depino 2015, Machado, Whitaker et al. 2015, Washington, Kumar et al. 2015), and behavioral data in models of developmental immune deficiency are limited. Furthermore, little is known about the effects of immune disorders on maternal behavior, although some studies have noted an effect (Hood, Dreschel et al. 2003, Meyer, Schwendener et al. 2006). Therefore, an

altered and poorly understood rearing environment may be interfering with the outcome and interpretation of behavioral results. In order to make a more direct comparison between the immune effects on behavior in animal models and the correlations between immune disruption and neurobehavioral disorders in humans we need more information on the immune impact on maternal behavior and the resulting behavior of the offspring. My research shows for the first time that the severe combined immunodeficiency (SCID) mice exhibit deficits in maternal behavior. This is of particular importance as this model has been used previously to assess behavior, but differences in rearing environment have not been take into account until now (Brynskikh, Warren et al. 2008, Derecki, Cardani et al. 2010). Furthermore, juvenile males have impaired social recognition, and replacement of healthy splenocytes rescues this behavior. On the other hand, social preference is more significantly impacted by maternal care than immune cell function. These results point to long term and specific effects of rearing environment and developmental immune deficiency on social behavior.

Endocrine disruptors have been a part of the environment for many years, and there are new compounds manufactured on a regular basis. These chemicals interfere with endogenous steroid hormone processes. Early life is a sensitive period for exposure, and changes made during this time can be long lasting and heritable to future generations. As more EDCs are created and new chemicals are put into use, in depth research is required to understand the consequences of exposure to these compounds. DEHP is an abundant EDC that has dose dependent effects on testosterone (Borch, Metzdorff et al. 2006, Do, Stahlhut et al. 2012) and aromatase (Andrade, Grande et al. 2006) and is linked to altered neurodevelopment, obesity, and steroid hormone changes (Maloney and Waxman 1999, Lovekamp-Swan and Davis 2003, Borch, Metzdorff et al. 2006, Larsen and Nielsen 2007, Desvergne, Feige et al. 2009, Gonzalez, Corton et al. 2009, Lin, Chen et al. 2011, Wood, Jokinen et al. 2014). Although new studies are being conducted, many of them still use doses much higher than the estimated average human consumption rate. Furthermore, exposure varies based on age and environment, making it essential to study a wide

range of doses (Silva, Barr et al. 2004, Crinnion 2010, Do, Stahlhut et al. 2012, Centers for Disease Control 2013). It is of fundamental importance that we understand what the human population is being exposed to on a daily basis, and the outcomes of that exposure in order to better evaluate the consequences for the present generation, and for those yet to come.

Several overarching questions regarding the impact of DEHP remain; does DEHP exposure have effects on behavior at low, human relevant doses? Do these results cross generations, and how does that compare to the correlations observed in humans? The research to date tells us that there are connections between DEHP metabolite levels and behavioral outcomes in humans. Yet, there are not animal studies to help more carefully explore the relationship between exposure to DEHP and behavior. Studies using a wide range of doses from very low to the highest levels of human exposure are the only way to differentiate between specific effects of these variable doses. I have addressed these issues by conducting studies on social and anxiety behavior, stress response, and reproductive system development in several doses across generations. My results show that there are dose specific effects on behavior, stress response, and development, and that some of these effects are transgenerational. Most notably, I observed an increase in exploring and decrease in side by side sitting in animals exposed to 400μg/kg in utero, and the opposite behavior two generations later, in mice completely removed from contact with DEHP. This is the first information on the direct and transgenerational effects of DEHP on social behavior in low doses. Currently, the lowest dose used to study social behavior is 30mg/kg (Lee, Chiang et al. 2015). Furthermore, I recorded lowered serum corticosterone in females from a lineage exposed to 200mg/kg DEHP but in mice generations removed from exposure; however, there were no changes in anxiety behavior. These results imply a complex relationship between DEHP, stress, and anxiety that has not been previously suggested. Studies in animals exposed to DEHP at similar doses report anxiety behavior (Carbone, Ponzo et al. 2013, Xu, Yang et al. 2014).

My data indicate that changes in anxiety are not transgenerational, but corticosterone levels relevant to acute stress exposure are.

Importantly, there are reproductive outcomes in people associated with DEHP exposure and metabolite levels. Again, the current research falls short in this area. Most experiments done in animals are done using doses much higher than typical human levels, and only account for changes following direct or in utero exposure to DEHP. My results demonstrate transgnerational consequences in high doses as well as very low doses. In males from a 150mg/kg DEHP lineage I saw transgenerational effects of decreased seminal vesicle weight and increased anogenital distance. I also recorded lower testes weights in F3 animals from a dose comparable to humans, but did not see results in F1 animals of this dose. This suggests that the associations between metabolite levels and low prenatal testosterone in people are due to transgenerational exposure or changes from continued exposure across generations.

In general, the human data is complicated and the existing data *in vitro* and *in vivo* are complex and indicative of a non-monotonic dose response. Studies in human relevant doses and research that spans multiple generations are currently lacking. This will shed more light on what specific amounts and particular time periods of DEHP are meaningful to study further. The studies in this dissertation are important and necessary first steps in addressing these issues and yield significant and interesting results.

Chapter II

Transgenerational effects of di-(2-ethylhexyl) phthalate, DEHP, on stress hormones and behavior

Introduction

Endocrine disrupting chemicals (EDCs) are abundant in the environment. One of the most prevalent of these compounds is di(2-ethylhexyl)phthalate (DEHP) with yearly production in the US estimated at 1-4 million tons (McKee, Butala et al. 2004, Halden 2010). Recently, levels of DEHP and its metabolites (primarily MEHP) have been higher than previously estimated in certain individuals (Loff, Kabs et al. 2000, Gaudin, Marsan et al. 2011, Mallow and Fox 2014) and careful examination of conversion from animal to human doses indicates relevance of high-dose studies (Reagan-Shaw, Nihal et al. 2008). DEHP is found in flexible plastic products, specifically polyvinyl chloride (PVC), food packaging, medical equipment, pill coatings and synthetic flooring (Latini, Verrotti et al. 2004). Exposure in humans is variable depending on age and environment (Crinnion 2010), but occurs on a daily basis; the vast majority of people tested have measurable levels of DEHP and/or MEHP in their urine. Furthermore, DEHP crosses the placenta (Singh, Lawrence et al. 1975), is absorbed through the skin (Hopf, Berthet et al. 2014), and metabolites are found in breast milk (Kim, Lee et al. 2015). Daily human intake levels have been estimated between 6 and 21mg/kg/day, with children at the higher end of this range.

Pregnancy levels of MEHP are correlated, in male infants, with decreased levels of testosterone, estradiol, progesterone, inhibin B and INSL3. High DEHP exposure in utero has also been linked with decreased play behavior in boys (Swan, Liu et al. 2010), and concentrations of DEHP metabolites in urine are higher in autistic as compared to control individuals (Testa, Nuti et al. 2012). Gestational DEHP exposure affects anxiety and depression-like behavior in animals (Carbone, Ponzo et al. 2013, Xu, Yang et al. 2014), and DEHP metabolites inhibit the RNA expression and activity of the corticosterone-inactivating enzyme 11-beta-hydroxysteroid dehydrogenase 2 (Hong, Li et al. 2009, Zhao, Chu et al. 2010). In 40-day old rats, oral administration of 750mg/kg DEHP for four days increases serum adrenocorticotropic hormone (ACTH) and corticosterone (Supornsilchai, Soder et al. 2007). These data indicate that DEHP

acts on several levels of the hypothalamic-pituitary-adrenal (HPA) axis. Transgenerational effects of DEHP, including disturbed germ cell association, lower sperm counts and decreased motility have been noted in mice three generations removed from initial exposure (Doyle, Bowman et al. 2013). Here, using similar doses and the same treatment regimens that produced transgenerational outcomes, we evaluated behavior and stress responses in F3 mice from DEHP-lineages along with their controls.

Materials and Methods

Mice: Breeding and treatments were conducted as described previous (Doyle, Bowman et al. 2013). Briefly, the F0 ancestors of these F3 mice (C57BL/6J) were treated with DEHP via oral gavage at a dose of 150 or 200mg/kg body weight/day during gestational days 7-14. Each animal was weighed daily and the weights used to calculate the amount of DEHP to reach the desired dose in 200μL of corn oil. Controls received 200μL of corn oil. Their offspring (F1) were bred and the F2 offspring bred again to create a third generation (F3) removed from initial DEHP exposure. Siblings were not paired. Our studies were conducted on F3 mice. All 150mg/kg mice, and 200mg/kg mice used for weights and anogenital measurements were bred and maintained at Washington State University. A second cohort of F2 200mg/kg mice were bred in Washington and then shipped to Virginia and used to create F3 offspring for additional behavioral testing at the University of Virginia. Mice were maintained at the University of Virginia School of Medicine, Jordan Hall Animal Facility. In Washington mice were maintained in the Experimental Animal Laboratory Building. The University of Virginia and Washington State University Animal Care and Use Committees approved all procedures used and described here. All animals were maintained on a 12:12 light/dark cycle and provided with food (Harlan Teklad diet 2918) and water *ad libitum*.

Social Interaction: Males in the 200mg/kg DEHP-lineage groups were used to examine juvenile social interactions. Methods were adapted from previous research (Cox and Rissman 2011). Mice were tested during the dark portion of the light cycle between 35-42 days of age with an

unfamiliar partner matched for age and lineage (Control Males N=6 pairs, 200mg/kg DEHP-lineage Males N=12 pairs). Members of each pair were individually habituated to an empty mouse box for 10 minutes, and then paired, placed in a second empty box, and observed for 10 minutes by an observer blind to their lineage. A scan sampling method was used; every 15 seconds the pairs were observed and behaviors recorded on a check sheet. Each pair was scored as a unit, with total behaviors from both individuals included. Social behaviors scored were: side-by-side sitting, approach, following, crawling, sniffing the other mouse, and social grooming. Non-social behaviors noted were exploring, self-grooming, digging, and sitting alone.

Elevated Plus Maze: This protocol was adapted from methods described previously (Scordalakes and Rissman 2004). Mice were tested during the light portion of the light cycle (150mg/kg between 25-32 days of age; 200mg/kg between 35-42 days of age) on the elevated plus maze (Columbus Instruments; wall height: 6", arm length: 11.75", arm width: 2"). An observer, blind to the group, scored each mouse for 5 minutes (150mg/kg Control-Lineage Males N=11, 150mg/kg DEHP-Lineage Males N=11, 150mg/kg Control-Lineage Females N=10, 150mg/kg DEHP-Lineage Females N=10, 200mg/kg Control-Lineage Males N=11, 200mg/kg DEHP-Lineage Males N=21, 200mg/kg Control-Lineage Females N=8, 200mg/kg DEHP-Lineage Females N=19). Time spent in the open and closed arms and the center was recorded.

Restraint Stress and Corticosterone: Mice from the 150mg/kg dose DEHP-lineage (and their controls) were used to assess corticosterone. Both sexes were used for this study. Mice were housed in pairs by sex and treatment for at least 10 days before the experiment. One mouse in each pair was randomly assigned to the stressed or baseline condition. The mice assigned to the stressed condition were restrained in a 50mL conical tube (with a nose hole at the tip) for 15 minutes immediately prior to sacrifice (Control-Lineage Females Stressed N=10, Control-lineage Females Baseline N=9, DEHP-lineage Females Stressed N=8, DEHP-lineage Females Baseline N=10, Control-lineage Males Stressed N=6, Control-lineage Males Baseline N=6, DEHP-lineage Males Stressed N=8, DEHP-lineage Males Baseline N=7). All mice were euthanized using CO2

followed by rapid cervical dislocation. Trunk blood was collected, placed on ice, centrifuged, serum collected and frozen. Pituitaries were removed and immediately frozen on dry ice. The University of Virginia Ligand Core analyzed serum corticosterone concentration using RIA; samples with sufficient volume were run in duplicate, when necessary, samples were run as singlets (35 samples). Samples with % coefficients of variation greater than 20% (2 samples) or values outside the reportable range of 9.0-836.9ng/mL (4 samples) were eliminated; of samples analyzed the mean CV was 4.8±0.55%.

Anogenital Distances and Organ Weights: At sacrifice, mice ranged between 29-30 days of age (150mg/kg lineage) and 38-43 days of age (200mg/kg lineage). We collected body weights, anogenital distances, uterine and seminal vesicle weights (150mg/kg Control Females N=19, 150mg/kg DEHP-lineage Females N=20, 150mg/kg Control Males N=13, 150mg/kg DEHP-lineage Males N=15, 200mg/kg Control Males N=11, 200mg/kg DEHP-lineage Males N=7).

Quantitative Real-Time PCR: We assessed mRNA concentrations in pituitaries from mice in the DEHP (150mg/kg) and control lineages for the following genes: Corticotrophin releasing hormone receptor (*Crhr1),* Guanyl nucleotide binding protein, alpha stimulating (*Gnas*). Real time PCR primers (Table 3) were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA). cDNA was synthesized from 80 ng of RNA using SS Vilo cDNA Synthesis Master Mix (Life Technologies, Foster City, CA) and used as template for real time PCR assays with an ABI 7500 Fast Real-Time PCR System (Life Technologies). The real time RT-PCR was conducted on two - three separate animal RNA preps, with each sample done in duplicate or triplicate. Threshold values (Ct) for the gene of interest and a reference gene, ribosomal *S2 (Rps2)* were determined using 7500 Software v2.0.1 (Life Technologies). The expression level of the gene of interest was evaluated using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001).

Statistical Analyses: Reproductive organ weights, mRNA concentrations and anogenital distances were analyzed with one- or two-way ANOVA. Behavioral data were analyzed with either one-way or two-way ANOVAs as appropriate following an Arcsine transformation of proportional social behavior data. Corticosterone data were analyzed with three-way ANOVA for all groups, and two-way ANOVA for separate analysis of stressed and baseline corticosterone levels. We used Fisher's LSD post-test for pair-test analyses.

Results

Social Interaction tests: Males from the 200mg/kg DEHP-lineage spent more time digging (**Figure 7**; F(1,18)=9.67; p<0.01) and less time self-grooming (**Figure 7**; F(1,18)=6.41; p<0.05) than control males. None of the other behavioral measures were significantly different.

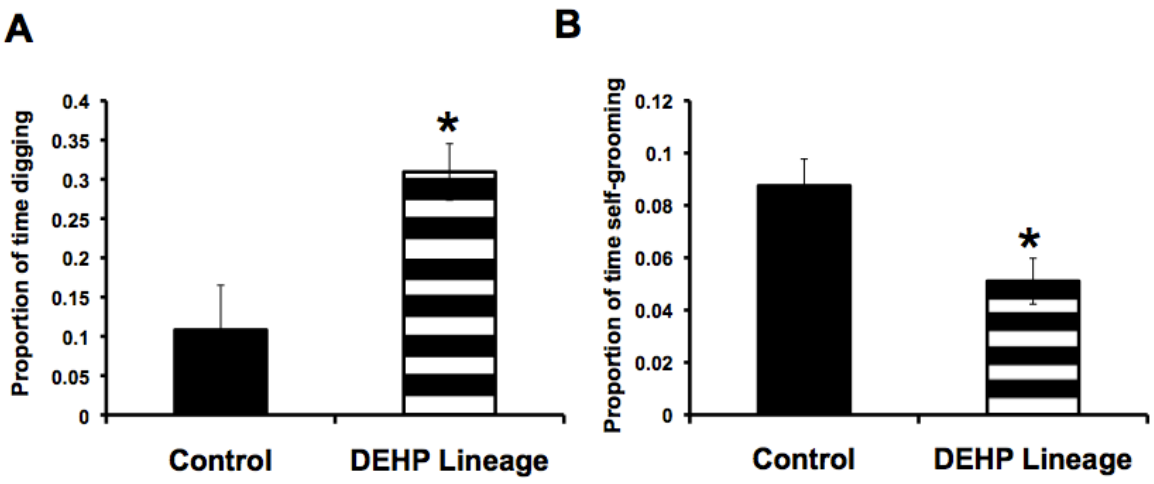

Figure 7: Means (±SEM) Proportion of time spent A) digging and B) self-grooming. Males from the DEHP (200mg lineage) displayed more digging and less self-grooming than their controls, *p<0.05. (Control Males N=6 pairs, 200mg/kg DEHP-lineage Males N=12 pairs)

Elevated Plus Maze: There were no significant effects of treatment or sex on behavior in the EPM (Table 1).

Time Spent in sections of the EPM	150 mg/kg Control-lineage Males	150 mg/kg DEHP-lineage Males	150 mg/kg Control-lineage Females	150 mg/kg DEHP-lineage Females	200 mg/kg Control-lineage Males	200 mg/kg DEHP-lineage Males	200 mg/kg Control-lineage Females	200 mg/kg DEHP-lineage Females
Open	45.27 ± 9.10	64.64 ±16.57	59.00 ± 12.27	57.50 ± 64.64	10.18 ± 3.83	10.67 ± 2.82	17.74 ± 7.89	12.35 ± 1.91
Middle	55.55 ± 5.23	52.18 ± 5.07	66.00 ± 5.75	54.10 ± 3.42	25.88 ± 4.77	31.25 ± 3.87	40.56 ± 6.22	35.48 ± 4.18
Closed	199.18 ± 15.30	183.18 ± 17.93	175.00 ± 15.31	188.40 ± 9.59	264.04 ± 7.98	258.20 ± 5.72	241.79 ± 12.79	252.27 ± 5.33

Table 1: There are no significant differences in elevated plus maze behavior between any groups.

Restraint Stress and Corticosterone: There was an overall effect of condition at the time of blood collection ($F(1,68)=534.46$; $p<0.00001$) and of lineage ($F(1,68)=6.77$; $p<0.01$) on corticosterone. Significant interactions between condition and sex ($F(1,68)=6.97$; $p<0.01$) and sex and lineage ($F(1,68)=6.96$; $p<0.05$) were noted. Because the effect of restraint stress (as predicted) was so large, to focus on the effects of sex and lineage we analyzed data from the stressed and baseline groups independently. Under baseline condition we observed a significant effect of sex ($F(1,35)=5.81$; $p<0.05$) wherein females had higher baseline corticosterone than males overall, and there was trend for an interaction between sex and lineage (**Figure 8A**; $F(1,35)=2.96$; $p<0.10$). When analyzing data from the stressed mice we observed a significant effect of

treatment (F(1,33)=5.05; p<0.05), and an interaction between sex and treatment lineage (**Figure 8B**; F(1,33)=4.44; p<0.05). Post hoc analyses showed that F3 females from the DEHP-lineage had lower serum corticosterone levels as compared to F3 control females and F3 control and DEHP-lineage males.

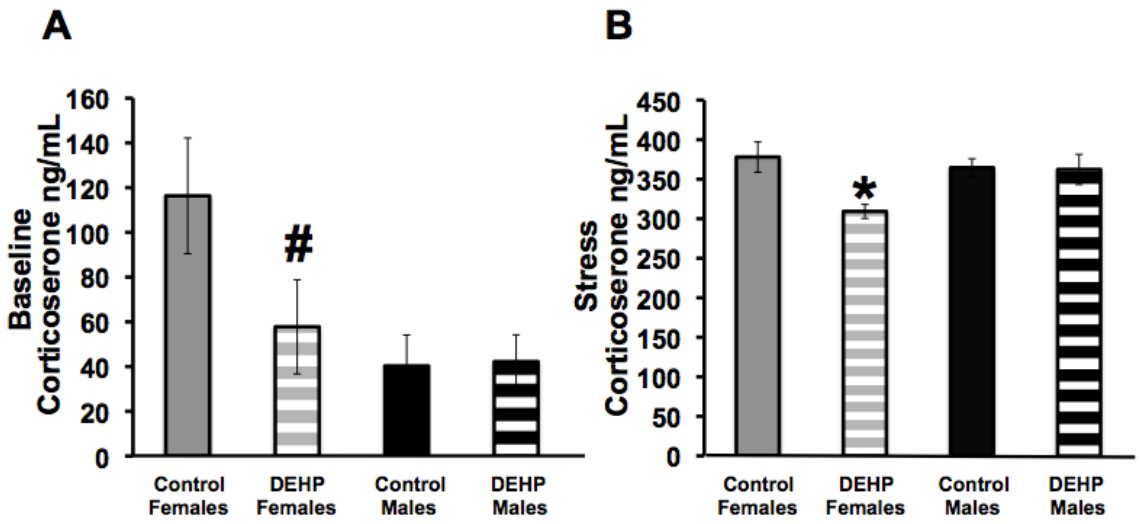

Figure 8: Mean (±SEM) corticosterone levels (ng/mL) measured in plasma from 150mg/kg DEHP-lineage females and controls. A) Under baseline conditions, DEHP-lineage mice tended to have lower corticosterone levels than all other groups (#p=0.10). B) After 15 minutes of restraint stress, DEHP-lineage females had lower corticosterone than control females *p<0.05. (Control-Lineage Females Stressed N=10, Control-lineage Females Baseline N=9, DEHP-lineage Females Stressed N=8, DEHP-lineage Females

Anogenital index and organ weights: Because sex differences in these measures are expected and are large, we analyzed each sex separately. In males, anogenital indices (anogenital distance/body weight) were significantly longer in 37-43 day old males from the 150mg/kg DEHP-lineage, as compared to their control males (Table 2; $F(1,30)=7.44$; $p<0.02$). DEHP-lineage 200mg/kg males had significantly lower body weights than controls (Table 2; $F(1,28)=6.27$; $p<0.02$). Seminal vesicle weights in F3 control mice were significantly heavier than in the DEHP (150mg/kg dose) group (Table 2; $F(1,28)=6.25$; $p<0.02$), but no differences were noted in the 200mg/kg groups. Females, regardless of lineage, had similar body weights, uterine weights, anogenital distances and anogenital indices (Table 2).

	150 mg/kg Control-lineage Males	150 mg/kg DEHP-lineage Males	150 mg/kg Control-lineage Females	150 mg/kg DEHP-lineage Females	200 mg/kg Control-lineage Males	200 mg/kg DEHP-lineage Males
Body Weight (g)	15.51 ± 0.28	15.15 ± 0.48	13.32 ±0.18	12.63 ± 0.39	24.63 ± 4.32	18.50 ± 0.78*
Anogenital Distance (mm)	11.28 ± 0.36	12.14 ± 0.33	5.74 ± 0.15	5.84 ± 0.32	13.67 ± 1.02	14.34 ± 0.74
Anogenital Index (mm/g)	0.73 ± 0.03	0.81 ± 0.02*	0.43 ± 0.01	0.45 ± 0.02	0.74 ± 0.02	0.77 ± 0.03
Seminal Vesicle Weight (mg)	7.15 ± 1.70	3.09 ± 0.34*	X	X	5.14 ± 0.56	4.90 ± 0.68
Uterine Weight (mg)	X	X	22.04 ± 1.10	20.22 ± 1.25	X	X

Table 2: Body weights, anogenital distances, anogenital indices, and reproductive organ weights collected from male and female F3 mice from either the150mg/kg or the 200mg/kg DEHP-and their control-s. Data were collected from mice ages 25-32 (150mg lineages) and 35-42 (200 mg lineages). *$p<0.05$, significantly different from the same sex control group. (150mg/kg Control Females N=19, 150mg/kg DEHP-lineage Females N=20, 150mg/kg Control Males N=13, 150mg/kg DEHP-lineage Males N=15, 200mg/kg Control Males N=11, 200mg/kg DEHP-lineage Males N=7)

Quantitative Real-Time PCR: To evaluate gene expression in the pituitary, we analyzed baseline and stressed conditions separately. Males and females were also analyzed separately since only females exhibited a significant change in serum corticosterone. In the baseline condition, we found a sex difference wherein males had *lower Crhr1* expression than females (**Figure 9A**; $F(1,11)=6.80$; $p<0.05$). There were no other significant differences in *Crhr1* in terms of sex or stress. In the stressed condition there was a sex difference wherein males had *higher Gnas* expression than females (**Figure 9B**; $F(1,12)=86.34$; $p<0.0001$). Separating the analysis by sex we found an interaction between stress and DEHP lineage in *Gnas* males (**Figure 9C**; $F(1,11)=9.01$; $p<0.05$) and females (**Figure 9D**; $F(1,12)=9.01$; $p<0.05$). Post hoc tests revealed that in both sexes the stressed animals from DEHP lineage had higher *Gnas* expression than non-stressed DEHP animals and both control groups. We did not detect any effects of sex, lineage or condition in any of the other genes measured.

Gene	Forward Primer	Reverse Primer
Rps2	CTGACTCCCGACCTCTGGAA	GAGCCTGGGTCCTCTGAACA
Crhr1	CGCAAGTGGATGTTCGTCT	GGGGCCCTGGTAGATGTAGT
Gnas	GGTAGCTGCTAGAGTCTACAGGAAYGG-TTTTTTGGTTGTGTGGTTATAGTTAGAG	CCATCAGTATGAGATAGCTCGATATATA-ACATATCAAAATAAACATCCAAACCT

Table 3: Primer sequences used in qRTPCR

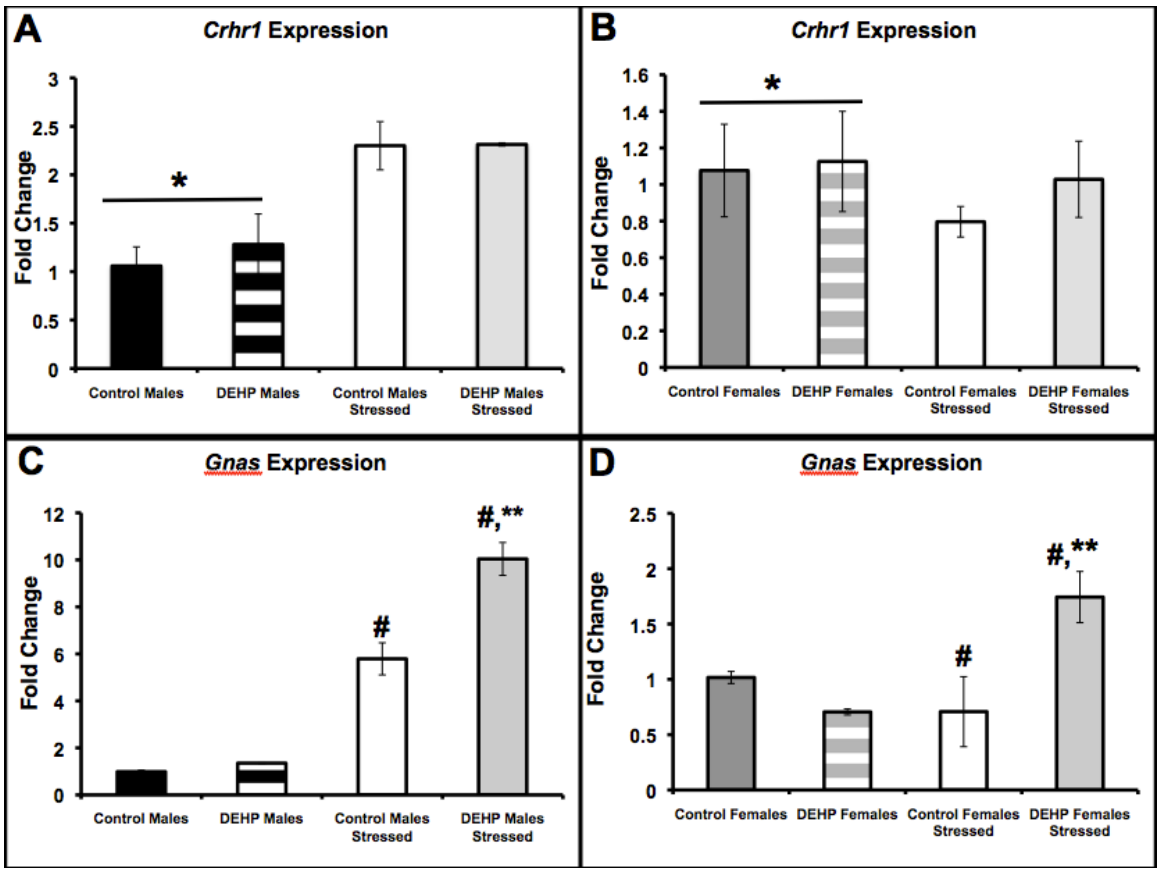

Figure 9: Mean (±SEM) mRNA expression levels in pituitaries from 150mg/kg DEHP-lineage and control mice. A) Males that were not stressed had lower *Crhr1* than B) females that were not stressed (*p<0.05) C) In males, stressed mice from the DEHP-lineage had higher *Gnas* mRNA than all other groups (**p<0.05). D) Likewise, restraint stressed females from the DEHP-lineage had higher *Gnas* expression that all other groups (**p<0.05). Overall, stressed males had higher *Gnas* than stressed females (#p<0.05). (Control-Lineage Females Stressed N=3, Control-lineage Females Baseline N=3, DEHP-lineage Females Stressed N=3, DEHP-lineage Females Baseline N=2, Control-lineage Males Stressed N=3, Control-lineage Males Baseline N=3, DEHP-lineage Males Stressed N=3, DEHP-lineage Males Baseline N=3)

Discussion

These data indicate, for the first time, that DEHP has transgenerational actions in both sexes on the neuroendocrine axes and behaviors. Intriguingly, the data suggest that the transgenerational target of DEHP is the HPA axis. In both sexes, expression in the pituitary of *Gnas* message was elevated by the combination of restraint stress and DEHP-lineage. Mice from the DEHP-lineage had normal basal levels, in males a 10-fold increase was found after restraint stress and in females we noted a smaller but still significant increase of 2-fold. In F3 females from the DEHP-lineage, restraint stress produced lower levels of corticosterone as compared to control mice. It is very possible that corticosterone was also modified by lineage in males, which we may have missed since only two collection time points were assessed.

In addition to these results we also observed lighter seminal vesicles in males from the 150mg/kg DEHP-lineage, which suggests they have lower testosterone levels as compared with peri-puberal control-lineage males. Since we did not find this same difference in males from the higher dose group, and these animals were sacrificed at an older age, we speculate that the DEHP-lineage males experienced delayed puberty. Interestingly, anogenital indices in F3 males from the 150mg/kg DEHP-lineage were longer than in the controls, although anogenital indices from the 200mg/kg DEHP-lineage were not changed.

Our behavioral results were limited to males and restricted to just two specific non-social behaviors, digging and self-grooming. Males in the 200mg/kg DEHP-lineage displayed less self-grooming but more digging than controls. Increased digging and self-grooming (both non-social behaviors) have been used as surrogate Autism-like phenotypes in mice (Crawley 2012). Until recently digging was considered an anxiety behavior (Masuda, Ishigooka et al. 2000). Yet, we did not find any differences in EPM behavior, the "gold standard" for anxiety (Neumann, Wegener et al. 2011). Previous research on EPM behavior in F1 DEHP-exposed animals has reported increased time in the closed arms of the EPM in pubertal male mice and rats (Carbone, Ponzo et al. 2013, Xu, Yang et al. 2014). In rats, this effect is ameliorated by testosterone administration

(Carbone, Ponzo et al. 2013). DEHP induces anxiety-like behavior in adolescent female rats as well, and this effect is counteracted by exercise (Wang, Chen et al. 2014).

In humans, a few studies have linked social behaviors (Lien, Ku et al. 2015) and neurodevelopmental disorders (Tellez-Rojo, Cantoral et al. 2013) to DEHP levels in blood and urine. DEHP metabolites in utero are also associated with Autism diagnosis (Testa, Nuti et al. 2012). Our hypothesis, based on data from boys who show reduced masculine play corresponding to DEHP metabolite levels in urine of pregnant mothers (Swan, Liu et al. 2010), was that male mice would spend less time than controls interacting with a novel partner. It is possible that if we had tested F1 mice we might have seen this effect. However, transgenerational effects of endocrine disruptors, in some cases, are not the same as effects noted in F1 offspring (Wolstenholme, Edwards et al. 2012). While not directly related to human neurobehavioral disorders, our data have shown that HPA axis activity, anxiety and stress responses are changed by DEHP. In vitro, MEHP regulates glucocorticoid activity by decreasing RNA levels and enzyme activity of 11-beta-hydroxysteroid dehydrogenase 2, the enzyme that inhibits corticosterone production (Hong, Li et al. 2009) and presumably leads to the increased corticosterone after DEHP exposure (Supornsilchai, Soder et al. 2007). Furthermore, corticosterone and *Gnas* results may also cause a complex set of changes that includes regulation of other steroid hormones yet to be measured. Testosterone levels are altered by DEHP in a non-monotonic dose response curve in pregnant mice and their offspring (Do, Stahlhut et al. 2012), and prenatal/neonatal sex hormones can influence the sensitivity of the HPA axis in adulthood (McCormick, Furey et al. 1998). Furthermore, adrenal aldosterone production in adult rats decreases following in utero exposure to DEHP (Martinez-Arguelles, Guichard et al. 2011). The mechanisms whereby DEHP has transgenerational effects on the HPA needs to be investigated.

Interestingly, we found expression changes in stress related genes in the pituitaries of both males and females. Due to previous studies showing changes in corticosterone, we hypothesized that gene expression changes in the pituitary would reflect upstream differences in

stress hormone pathways. A sex difference was noted in *Crhr1*where females had higher mRNA expression than males. Although there were no significant differences between DEHP and controls when females were analyzed separately, it is possible that elevated *Crhr1* is additive with *Gnas* and is related to sex differences specific role in corticosterone. *Gnas* transcribes a G protein α-subunit that couples hormone receptors, including adrenocorticotropic hormone (ACTH), through protein kinase A (Kozasa, Itoh et al. 1988, Weinstein, Xie et al. 2007). Previous research has shown that mouse adrenal cells treated with ACTH accumulate Gnas protein (Schimmer and Cordova 2015). Furthermore, Gnas is maternally imprinted in most tissues, with limited paternal imprinting. Mutation on the maternal allele leads to early lethality, obesity, and hormone resistance; paternal allele mutation precedes obesity and insulin resistance (Weinstein, Xie et al. 2007). We hypothesize that DEHP administration during gestational days 7-14 causes a heritable epigenetic change in *Gnas*. This would be an exciting discovery as the specific genes and mechanisms underlying transgenerational actions are just beginning to be examined (Rissman and Adli 2014).

Anogenital distance is one of the primary targets of DEHP, and, in this regard the doses we used should be "anti-androgenic" and produce smaller anogenital distances in exposed male rodents. (Gray, Ostby et al. 2000, Moore, Rudy et al. 2001, Do, Stahlhut et al. 2012) and humans (Swan, Main et al. 2005, Suzuki, Yoshinaga et al. 2012). Previous work in mice with similar doses shows a decrease in anogenital distance in neonatal F1 males that is not passed on to F2 and F3 generations (Doyle, Bowman et al. 2013). Our results, larger AGD in the F3 150mg/kg DEHP-lineage males at puberty are unexpected, but it was not observed in the F3 150mg/kg DEHP-lineage males. Moreover, F1 and F3 effects of endocrine disrupting compounds need not be the same and in fact are often reversed, and the age could also be a factor (Wolstenholme, Edwards et al. 2012).

Another androgen target tissue, the seminal vesicles, were lighter in DEHP-lineage males a finding more compatible with the presumed anti-androgenic actions of DEHP in F1 studies.

These two measures (ADG and seminal vesicles) are indicative of androgen exposures during different critical periods, in utero versus during puberty. In fact, we only observed lineage differences in males collected during puberty, which might be indicative of differences in the timing of pubertal onset. Interestingly, none of the pituitary genes in the HPG axis that we measured were effected by lineage.

Mechanisms for the differences reported here, in the absence of direct DEHP exposure include epigenetic changes such as DNA methylation, histone modifications or other potentially inherited regulators of gene transcription. Exposure to DEHP in utero produces increased global DNA methylation and increased expression of Dnmts in fetal testes (Wu, Zhu et al. 2010). Our F1 mice were treated with DEHP during a sensitive period in embryonic development, when demethylation and remethylation is taking place (Durcova-Hills and Capel 2008). Earlier work identifies this period of exposure to DEHP as one that results in lowered sperm count, and disrupted germ cell association in F3 mice (Doyle, Bowman et al. 2013). This study takes this model further by providing the first transgenerational evidence for changes in behavior and stress responses that we speculate are caused by epigenetic effects on genes such as *Gnas,* which are part of the HPA axis.

Chapter III

Direct and transgenerational dose response effects of di-(2-ethylhexyl) phthalate, DEHP, on hormones and behavior

i. Introduction

Man-made endocrine disrupting chemicals (EDCs) are abundant in the environment. One of the most prevalent compounds is Di(2-ethylhexyl) phthalate (McKee, Butala et al. 2004, Halden 2010). Exposure in humans is variable depending on age and environment (Crinnion 2010), but the vast majority of people tested have measurable levels of DEHP and its metabolites in urine. Average daily human intake has been estimated between 6 and 21 μg/kg/day, with children at the higher end of this range. Furthermore, DEHP crosses the placenta (Singh, Lawrence et al. 1975), is absorbed through the skin (Hopf, Berthet et al. 2014), and it's metabolites are found in human breast milk (Kim, Lee et al. 2015). DEHP is used in production of flexible plastics, specifically polyvinyl chloride (PVC) food packaging, medical equipment, medical pill coatings and synthetic flooring (Latini, Verrotti et al. 2004).

Some behavioral outcomes in humans have been associated with early life exposure to DEHP. For example, decreased masculine play in boys (Swan, Liu et al. 2010), is correlated with higher concentrations of DEHP metabolites in mother's urine during pregnancy. In autistic children, DEHP metabolites are elevated as compared to control individuals (Testa, Nuti et al. 2012). Gestational DEHP exposure increases anxiety and depression-like behavior in animals (Carbone, Ponzo et al. 2013, Xu, Yang et al. 2014). At some doses this anxiety phenotype is rescued in males with peripheral testosterone injection (Carbone, Ponzo et al. 2013). However, at higher doses, anxiety behavior is specific to females (Xu, Yang et al. 2014), demonstrating the significance of different doses and different responses in males and females. There are direct effects of DEHP metabolites on glucocorticoids (Hong, Li et al. 2009), suggesting that anxiety behavior is a secondary effect of stress hormones. Recent data from our lab shows that mice, three generations removed from gestational DEHP exposure, have altered social behavior and that females have lower levels of corticosterone prior to and following restraint stress as compared with controls (Quinnies et al in press).

The majority of the actions of DEHP examined to date have been in the reproductive system. Typically DEHP has been classed as an antiandrogen, and in both rodents and humans high gestational DEHP metabolite levels correlate with decreased anogenital distance in males (Moore, Rudy et al. 2001, Swan, Main et al. 2005, Do, Stahlhut et al. 2012, Bornehag, Carlstedt et al. 2015). However, much like other endocrine disruptors, DEHP has non-monotonic dose response actions. In mice, high doses cause a drop in maternal and fetal testosterone compared to controls, whereas lower levels of DEHP elevate testosterone (Do, Stahlhut et al. 2012). Maternal levels of MEHP are correlated with decreased levels of testosterone, estradiol, progesterone, as well as Sertoli cell product inhibinB and Leydig cell product INSL3 in male infants.

Transgenerational effects of DEHP in mice include disturbed germ cell association, lower sperm counts and decreased motility (Doyle, Bowman et al. 2013). Using similar doses and treatment paradigms, we have shown that F3 males have decreased seminal vesicle weight and increased anogenital distances, as compared with control males (Quinnies et al in press). Here, we tested the impact of a range of human relevant doses given to mice on social and anxiety behaviors. We evaluated behavioral and reproductive effects of F1 males and females exposed to DEHP *in utero*, and in F3 offspring . We noted significant changes in social behavior, anxiety behavior, anogenital distance and testes weights. This is the first time that social behavior changes in response to DEHP have been observed at these doses and the first time that transgenerational effects have been recorded following low-dose exposure to DEHP.

ii. Materials and Methods

Animals: Male and female C57BL/6J mice were housed and bred at the University of Virginia according to the guidelines of the Animal Care and Use Committee. All animals were maintained on a 12:12 light/dark cycle and provided with a low phytoestrogen diet (Harlan Laboratories, Indianapolis, IN #2918) and water *ad libitum*. Adults were paired with males and shortly after lights on each day (1300 hrs) females were checked for mating plugs. When a plug was observed,

males were removed, females remained singly housed and were given 50mL of corn oil containing 0, 5, 40, or 400 μg/kg/day of DEHP daily during the light portion of the cycle. The DEHP/corn oil mixture was presented in a Cocoa Puff placed in the dam's cage. Two to three females that plugged on the same day were fed the same DEHP dose to acquire synchronized births for social behavior testing. Starting 18 days after the plug was found, dams were checked daily for pups. On P1 (the day after birth, postnatal day 1) anogenital distances and body weights of the entire litter was recorded. The litters were culled to 5-7 pups each with as close as possible to a balanced sex ratio. Dams continued to receive their daily cocoa puff with DEHP with the final one presented on P10. The pups were weaned on P21 and body weighs recorded on P28. A subset of the mice were used for behavioral testing, and sacrificed as adults by rapid cervical dislocation following anesthesia by isoflourane. Upon sacrifice body weights, testes, seminal vesicle, and uterine weights were collected. Another subset was used for breeding. These F1 females were paired with dose-matched males from different parents to create F2 mice. The same breeding protocol was followed with the F2 mice to create F3 mice. P1 measurements were repeated in F2 and F3 animals. All behavioral testing and measurements done in the F1s were repeated in the F3 generation.

Maternal Behavior: We assessed maternal behavior to explore the hypothesis that DEHP might change maternal care. Both F0 dams (mothers to F1 offspring) and F2 dams (mothers to F3 offspring) were observed in their home cages for 30 minutes, once a day during the dark on P2, 4 and 6. We used scan-sampling methods to record maternal behaviors. We observed the nest every 15-seconds, recorded all behaviors, and noted if the dam was on or off of the nest. On the nest behaviors were: licking and grooming pups, self-grooming, active nursing, passive nursing, nest building and hovering. Behaviors off of the nest were eating/drinking, self-grooming, and digging/climbing. These methods were adapted from previous research (Chourbaji, Hoyer et al. 2011). Values were analyzed as proportions of total observations.

MEHP analysis: A separate cohort of dams was paired, checked daily for plugs, and given a daily dose of DEHP as previously described. These dams were sacrificed at E18 (embryonic day 18) by rapid cervical dislocation following anesthesia by isoflourane. Trunk blood was collected from the dams and pups and serum frozen. Serum was pooled by dose and assayed for MEHP levels. Standard curve spiking solutions were prepared in methanol. The standard curve for LC/MS/MS analysis consisted of concentrations ranging from 1 to 1000 ng/mL. Internal standard (MEHP-d_4) was added at 5 μL per sample from a solution in methanol at a concentration of 1 μg/mL. 50 μL of serum was aliquoted into a polypropylene microfuge tube. 150 μL of methanol was added and 5 μL of internal standard and 5 μL methanol were added. Samples were vortexed, centrifuged (13000 rpm for 10 minutes) and 50 μL of the supernatant was transferred to a glass Agilent LCMS low volume insert and mixed with 50 μL of 5 mM Ammonium Acetetate. For standards, 50 μL of blank mouse serum was mixed with 5 μL of spiking solution containing each compound and extracted as per standards. Standard curves were run prior to mouse serum samples and after mouse serum samples. Quality control samples were included in the middle of the analysis. Standards were within 15% of nominal or 20% at LOQ.

Social interaction task: Two mice matched for age (P28-32), sex, and treatment from different litters were habituated to new individual cages for 10 minutes, then, recorded as a pair in a clean cage for 30 minutes. Behaviors were scored from the video. Every 15 seconds an observation was taken and the behavior of each mouse, identifiable by a tail stripe, was recorded as one event. Examples of social behaviors include: side-by-side sitting, grooming partners, or acts of nose-to-nose or anogenital sniffing. Non-social behaviors: exploring the cage, self-grooming, and sitting alone. This test was done during the dark portion of the light cycle.

Elevated Plus Maze: Mice were tested as juveniles (P30-35), and behavior was recorded for 5 minutes. This test was conducted as previously reported by the lab (Imwalle, Gustafsson et al. 2005). The mice were habituated to the behavioral test room for at least 30 min prior to testing. To begin the test each mouse was placed on the center of the plus maze. The total time spent in

the closed and open arms and the numbers of crosses through the middle were recorded and subsequently scored from video. This test was conducted during the dark portion of the light cycle between 0900-1200.

Statistical Analysis: All analysis was done using NCSS 2007. Social interaction and maternal behavior data were expressed as a percentage of total activity following the scan sampling data that resulted in a set of discrete numbers. To normalize these data we used an arcsine transformation. The transformed social behavior and elevated plus maze data were analyzed by two-way ANOVA, the maternal behavior data were analyzed by repeated measures ANOVA. The weights and anogenital measurements as well as the MEHP data were analyzed by one-way ANOVA. All ANOVAs were followed by Fisher's LSD post-test to evaluate pairwise interactions.

iii. Results

Maternal Behavior: The exposure model that we used was meant to closely replicate human conditions, and did not allow for fostering of pups. Therefore, we hypothesized that DEHP exposure may affect maternal behavior and, if this were the case, that the behavior in the offspring may be due to differences in rearing. However, there were no significant differences in time spent on the nest, licking/grooming, or nursing between control dams and dams receiving different doses of DEHP in the F0 dams (mothers to F1 offspring; Control N=27, 5μg/kg N=19, 40μg/kg N=24, 400μg/kg N=17) or F2 dams (mothers to F3 offspring; Control N=14 5μg/kg N=14 40μg/kg, 400μg/kg N=17) (**Table 6**). This led us to the conclusion that this is a sound model for consistency with human conditions as well as remaining appropriate for measuring behavior in offspring.

	Time on Nest	Time licking/ grooming	Time Nursing
F1 Control Dams	0.60±0.09	0.23±0.04	0.26±0.07
F1 5µg Dams	0.66±0.10	0.25±0.03	0.33±0.10
F1 40µg Dams	0.62±0.09	0.30±0.04	0.20±0.07
F1 400µg Dams	0.67±0.09	0.25±0.04	0.24±0.09
F3 Control Dams	01.03±0.09	0.38±0.02	0.67±0.09
F3 5µg Dams	0.94±0.12	0.34±0.07	0.54±0.14
F3 400µg Dams	0.74±0.10	0.34±0.05	0.30±0.09

Table 4: There are no significant differences in maternal behavior across doses.

MEHP analysis: Many of the serum samples that we collected (N=6 for all doses) had metabolite levels below detection. Of the serum that we collected from dams only the 400µg/kg group had detectable MEHP and 5-OH-MEHP in all measured samples with an average of 160ng/mL of MEHP and 1.6 ng/mL of 5-OH-MEHP. Of the serum that we collected from embryos only the E18.5 embryos from the 400µg/kg dose had detectable MEHP and 5-OH-MEHP with an average of 96.7ng/mL of MEHP and 1 ng/mL of 5-OH-MEHP.

Social interaction task: We observed sex-specific, dose-specific, and sex and dose interaction changes in both F1 and F3 social behavior. In F1 mice (Control Males N=18, Control Females N=14, 5µg/kg Males N=14, 5µg/kg Females N=14, 40µg/kg Males N=6, 40µg/kg Females N=12, 400µg/kg Males N=8, 400µg/kg Females N=12) we found a sex effect ($F(1,98)=6.53$; $p<0.05$) and dose effect ($F(3,98)=3.50$; $p<0.05$) in time spent exploring where females explored more and 400µg/kg animals spent more time exploring than control animals (**Table 5, Figure 10A**). We

also observed a dose effect ($F(3,98)=7.15$; $p<0.01$) and an interaction ($F(3,98)=4.94$; $p<0.01$) in time spent side-by-side sitting where 40 and 400μg/kg mice spent less time side-by-side sitting than control animals and control males spent more time side-by-side sitting than 40 and 400μg/kg males and 40μg/kg and control females (**Figure 10B, Table 5**). There was also a dose effect ($F(3,98)=5.96$; $p<0.01$) and an interaction ($F(3,98)=3.88$; $p<0.05$) in time spent investigating the other animal where 40μg/kg animals spend more time investigating than all other doses and 5 and 40μg/kg males and 40μg/kg females spend more time than control males and control females (**Figure 10C, Table 5**). Furthermore, we observed a dose effect ($F(3,98)=2.40$; $p<0.05$) and an interaction ($F(3,98)=3.86$; $p<0.05$) in time spent grooming where the 40 and 400μg/g doses spend more time grooming than controls and 400μg/kg males groom more than control and 5μg/kg males, and control and 5 and 400μg/kg females (**Figure 10D, Table 5**). In F3 mice (Control Males N=10, Control Females N=6, 5μg/kg Males N=8, 5μg/kg Females N=12, 400μg/kg Males N=4, 400μg/kg Females N=6) we found a dose effect ($F(2,46)=9.89$; $p<0.01$) in time spent exploring 400μg/kg animals spent more time exploring than control animals (**Figure 11A, Table 5**). We also observed a sex effect ($F(2,46)=6.85$; $p<0.05$), dose effect ($F(2,46)=4.56$; $p<0.05$) and an interaction ($F(2,46)=5.67$; $p<0.01$) in time spent side-by-side sitting where males spend more time side-by-side sitting than females, 400μg/kg animals spend more time side-by-side sitting than controls, and 400μg/kg males spend more time than control and 5μg/kg males and control, 5 and 400μg/kg females (**Table 5, Figure 11B**). Additionally, we found a sex effect ($F(1,46)=5.80$; $p<0.05$), dose effect ($F(2,46)=10.10$; $p<0.01$) and an interaction ($F(2,46)=3.37$; $p<0.05$) in total time spent interacting socially where males spend more time interacting than females, 400μg/kg animals spend more time interacting than controls, and 400μg/kg males interact more than control and 5μg/kg males and control, 5 and 400μg/kg females (**Table 5, Figure 11C**). We also found a sex effect ($F(1,46)=5.80$; $p<0.05$), dose effect ($F(2,46)=10.10$; $p<0.01$) and an interaction ($F(2,46)=3.37$; $p<0.05$) in total time spent exhibiting non-social behavior (**Table 5, Figure 11D**).

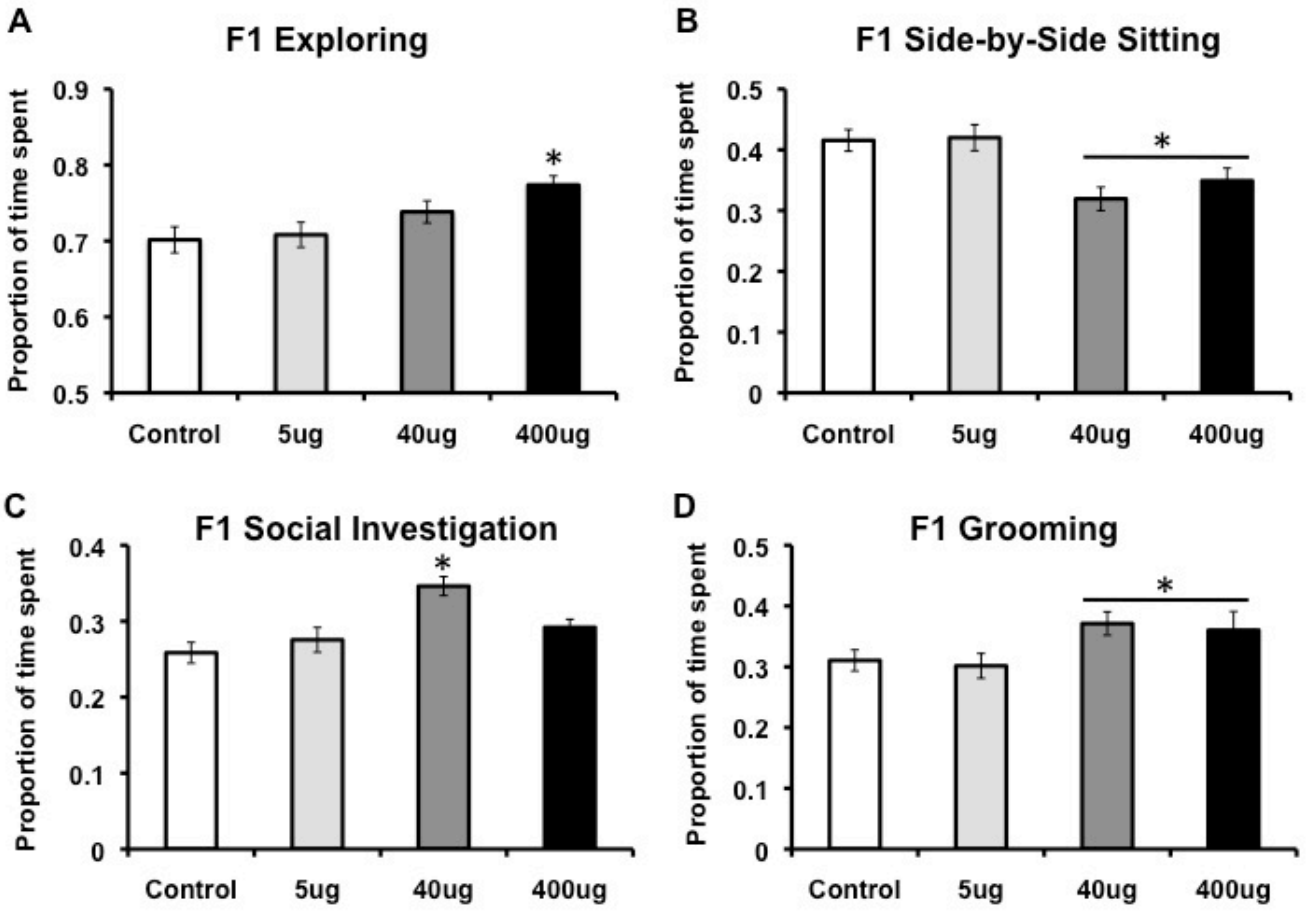

Figure 10: A) In F1 mice we found a sex effect and dose effect in time spent exploring where females explored more and 400μg/kg animals spent more time exploring than control animals. B) 40 and 400μg/kg mice spent less time side-by-side sitting than control animals and control males spent more time side-by-side sitting than 40 and 400μg/kg males and 40μg/kg and control females. C) 40μg/kg animals spend more time investigating than all other doses and 5 and 40μg/kg males and 40μg/kg females spend more time than control males and control females. D) 40 and 400μg/g doses spend more time grooming than controls and 400μg/kg males groom more than control and 5μg/kg males, and control and 5 and 400μg/kg females.

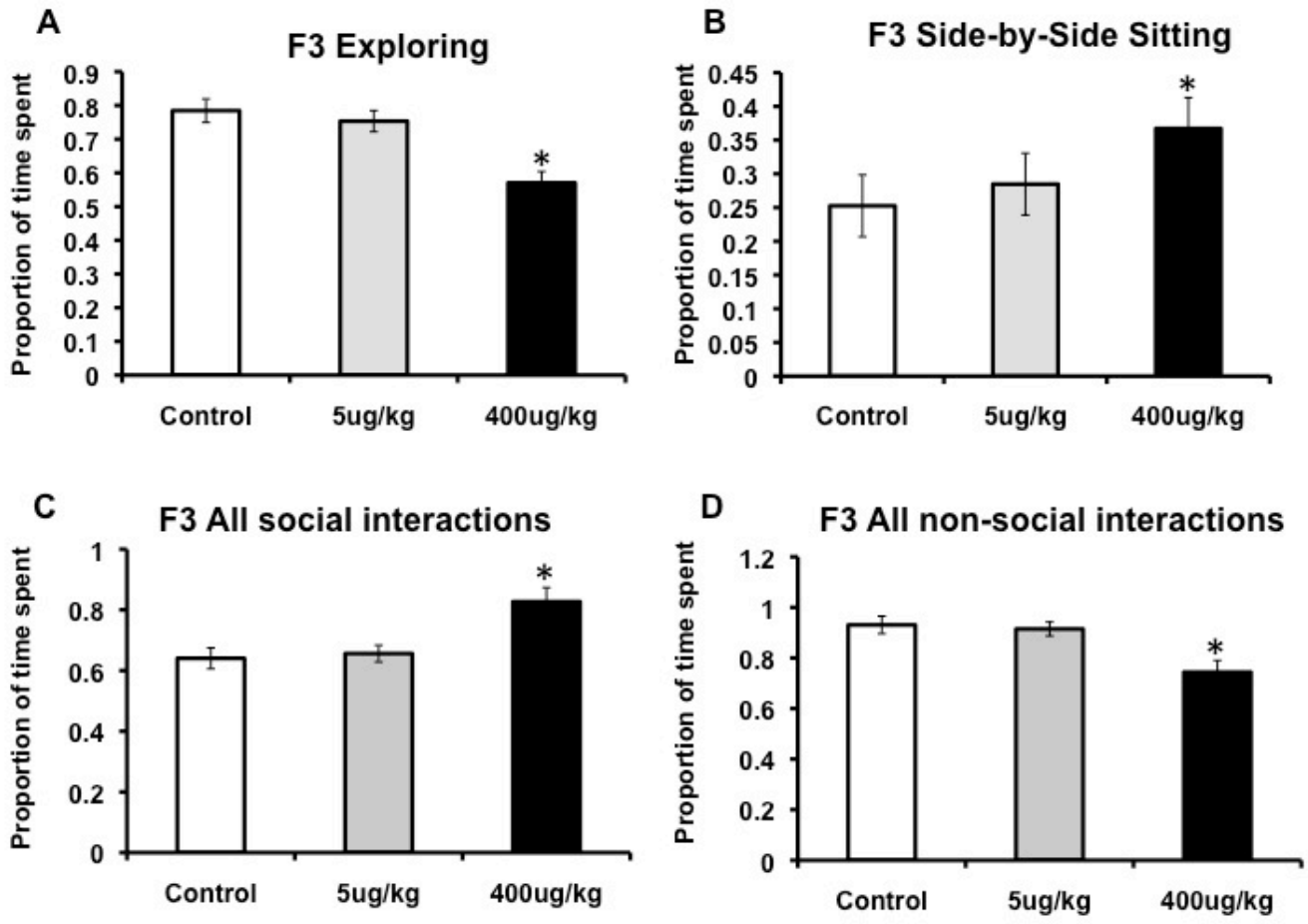

Figure 11: A) 400µg/kg animals spent less time exploring than control animals. B) Males spend more time side-by-side sitting than females, 400µg/kg animals spend more time side-by-side sitting than controls, and 400µg/kg males spend more time than control and 5µg/kg males and control, 5 and 400µg/kg females C) Males spend more time interacting than females, 400µg/kg animals spend more time interacting than controls, and 400µg/kg males interact more than control and 5µg/kg males and control, 5 and 400µg/kg females, and the D) reverse is true in non-social behaviors.

	Exploring	Side-by-side sitting	Social Investigation	Grooming	Total social behavior	Total non-social behavior
F1 Control Males	0.68±0.02^{a}	0.44±0.03^{a}	0.26±0.02^{a}	0.32±0.02	NS	NS
F1 Control Females	0.73±0.03$^{b,+}$	0.36±0.01^{b}	0.25±0.02^{a}	0.30±0.03^{b}	NS	NS
F1 5µg Males	0.67±0.71	0.46±0.02	0.32±0.02	0.27±0.02^{b}	NS	NS
F1 5µg Females	0.74±0.02^{+}	0.38±0.04	0.23±0.02	0.33±0.03^{b}	NS	NS
F1 40µg Males	0.71±0.02^{b}	0.27±0.01^{b,*}	0.34±0.02^{b,*}	0.40±0.05*	NS	NS
F1 40µg Females	0.75±0.02^{+}	0.34±0.03^{b,*}	0.35±0.01^{b,*}	0.35±0.01*	NS	NS
F1 400µg Males	0.77±0.02^{b,*}	0.29±0.04^{b,*}	0.27±0.01	0.44±0.06^{a,*}	NS	NS
F1 400µg Females	0.78±0.02*	0.39±0.02*	0.30±0.02	0.30±0.02^{b,*}	NS	NS
F3 Control Males	0.83±0.04	0.29±0.44$^{b,+}$	NS	NS	0.64±0.05$^{b,+}$	0.92±0.05^{b}
F3 Control Females	0.71±0.06	0.19±0.06^{b}	NS	NS	0.63±0.06^{b}	0.94±0.06$^{b,+}$
F3 5µg Males	0.79±0.06	0.25±0.03$^{b,+}$	NS	NS	0.67±0.05$^{b,+}$	0.90±0.05^{b}
F3 5µg Females	0.73±0.04	0.31±0.03^{b}	NS	NS	0.65±0.03^{b}	0.92±0.03$^{b,+}$
F3 400µg Males	0.49±0.04*	0.53±0.10$^{a,*,+}$	NS	NS	0.98±0.04$^{a,*,+}$	0.59±0.04^{a,*}
F3 400µg Females	0.62±0.32*	0.26±0.02$^{b,*,+}$	NS	NS	0.73±0.03^{b,*}	0.84±0.03$^{b,*,+}$

Table 5: All In each column and dose, different letters are significantly different from each other showing an interaction, + indicates a sex difference with annotated sex exhibiting more behavior, * indicates dose effect different from control group.

Elevated Plus Maze: In F1 mice (Control Males N=13, Control Females N=9, 5µg/kg Males N=8, 5µg/kg Females N=13, 40µg/kg Males N=5, 40µg/kg Females N=9, 400µg/kg Males N=5, 400µg/kg Females N=4) we found a sex effect ($F(1,61)=5.01$; $p<0.05$) and dose effect ($F(3,61)=2.79$; $p<0.05$) in time spent in the middle and open arm where the 5 and 40µg/kg animals spend less time outside of the closed arms than control mice (**Figure 12**). We observed did not note any dose effects of time spent in any portion of the maze in F3 animals.

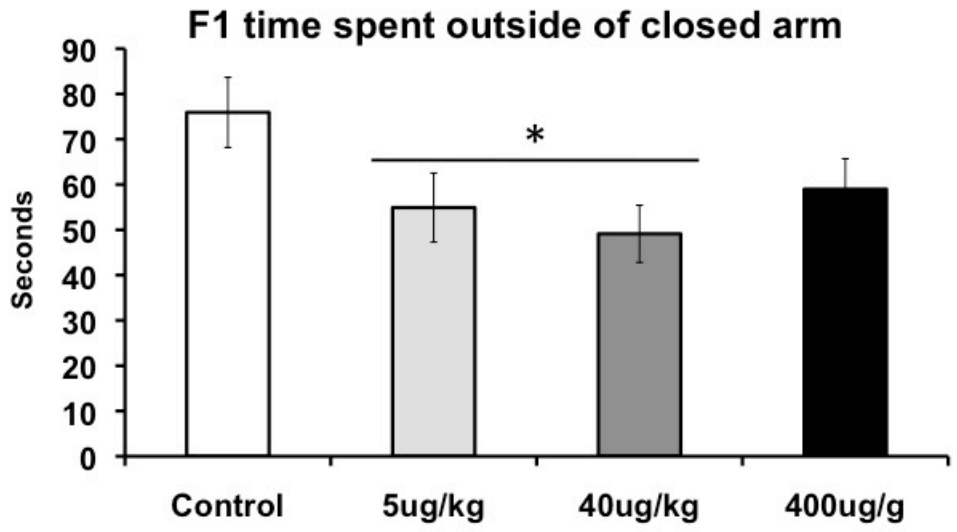

Figure 12: 5µg/kg and 40µg/kg animals spend less time outside of the closed arms than control mice.

Anogenital Distances and Organ Weights: There were no significant differences in anogenital index (anogenital distance/body weight) measurements in F1 or F2 pups other than the expected sex differences where males had larger anogenital indices than females. There were no differences observed in body weight in F1 or F2 pups. However, in F3 pups, we observed a sex effect ($F(1,140)=4.41$; $p<0.05$) with males (Control N=19, 5µg/kg N=29 40µg/kg N=9, 400µg/kg N=16) weighing more than females (Control N=24, 5µg/kg N=16, 40µg/kg N=4, 400µg/kg N=23) and a dose effect on body weight ($F(3,140)=7.45$; $p<0.001$) with the 5 and 40µg/kg doses weighing less than control animals (**Table 6**). Due to males and females being statistically different in body weight, we also analyzed the sexes individually. There was a dose effect on body weight in females when males and females were analyzed separately ($F(3,67)=6.55$; $p<0.001$) with the 5 and 40µg/kg doses weighing less than control females (**Table 6**). There was also a difference in anogenital index (anogenital distance/body weight, AGI) in females ($F(3,67)=2.7$; $p<0.05$) with 5µg/kg females having a higher anogenital index than control and 400µg/kg females (**Table 6**). In F1 males and females there were no differences in weights of testes, seminal vesicles, or uterine weights, but in F3 males (Control N=11, 5µg/kg N=12, 40µg/kg N=8, 400µg/kg N=14) we recorded a difference in testes weight/body weight where 5µg/kg animals are lower than all other doses including control animals ($F(3,45)=6.40$; $p<0.01$) (**Figure 13**).

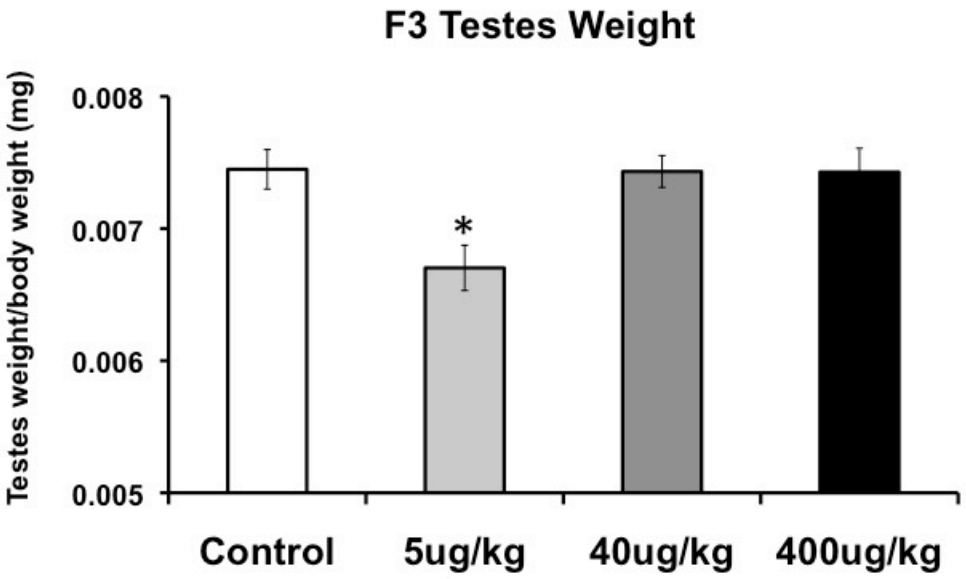

Figure 13: 5µg/kg animals have lower testes weight than all other doses including control animals.

	Control Males	Control Females	5ug/kg Males	5ug/kg Females	40ug/kg males	40ug/kg Females	400ug/kg Males	400ug/g Females
F1 Body Weight	1.50±0.11	1.40±0.06	1.36±0.02	1.30±0.03	1.35±0.03	1.36±0.02	1.31±0.03	1.37±0.03
F3 Body Weight	1.31±0.03	1.32±0.02	1.28±0.02	1.20±0.03*	1.20±0.05	1.11±0.13*	1.35±0.03	1.29±0.02
F1 AGI	1.03±0.07	0.68±0.05	1.03±0.02	0.68±0.03	1.06±0.03	0.66±0.03	1.07±0.07	0.60±0.04
F3 AGI	1.07±0.04	0.646±0.02	1.09±0.02	0.71±0.04*	1.06±0.04	0.75±0.01	1.02±0.03	0.65±0.01

Table 6: F3 Females from the 5µg/kg and 40µg/kg have lower body weight than controls. F3 Females from the 5µg/kg group have an increased anogenital index from the control group. * indicates significant difference from the control group.

iv. Discussion

Here we report that DEHP, a common endocrine disruptor with known effects on reproductive development and behavior in humans, has dose and sex-dependent effects on social behavior, anxiety behavior, and reproductive development in mice. Social behavior results are extensive, showing that mice from the 400μg/kg dose spent more time exploring than controls in the F1 and less time in the F3 generation. Interestingly, in the F1 generation 40 and 400μg/kg mice spent less time side-by-side sitting than control animals, and more specifically, 40 and 400μg/kg males and 40μg and control females spent less time side by side sitting than control males. However, in the F3 generation 400μg/kg animals spent more time side-by-side sitting than controls, and 400μg/kg males spend more time than control and 5μg/kg males and control, 5 and 400μg/kg females. In the F1 generation 40μg/kg animals spent more time socially investigating via sniffing than all other doses and 5 and 40μg/kg males and 40μg/kg females spent more time sniffing than control males and control females. Additionally, 40 and 400μg/kg mice spent more time grooming than controls and 400μg/kg males groomed more than control and 5μg/kg males and control and 5 and 400μg/kg females. We did not observe these investigation and grooming behaviors in the F3 animals. We did see an overall effect of all social behavior in the F3 animals that we did not observe in the F1 mice. Animals from the 400μg/kg dose spent more time interacting than controls, and 400μg/kg males interacted more than control and 5μg/kg males and control, 5μg/kg and 400μg/kg females. We also observed increased anxiety behavior in the 5 and 40μg/kg doses in the F1 generation but not in the F3 generation. Moreover, we saw effects on anogenital distance, body weight, and testes weight. In the F3 mice, 5 and 40μg/kg doses weigh less than control females. There was also a difference in anogenital index in females with 5μg/kg females having a higher anogenital index than control and 400μg/kg females Finally, F3 males of the 5μg/kg dose have lower testes weights than controls and the other doses.

These social behavior results substantially expand on the current literature in terms of information on dose response and specific behaviors. In humans, social behavior and

neurodevelopmental disorders have been linked to DEHP levels in blood and urine (Tellez-Rojo, Cantoral et al. 2013, Lien, Ku et al. 2015), DEHP metabolites *in utero* are associated with Autism diagnosis (Testa, Nuti et al. 2012), and reduced masculine play in boys (Swan, Liu et al. 2010). Boys with ADHD also have higher levels of DEHP metabolites, which are negatively correlated with cortical thickness (Park, Lee et al. 2015). The human data is complex, but there is a lack of animal studies on social behavior. Animal research on social behavior is needed to clarify specific outcomes at different doses and exposure timelines that humans experience. In this study we show that there are significant changes in the social behavior of the animals exposed to DEHP *in utero* as well as F3 offspring removed entirely from contact with DEHP. Based on the human masculine play data, we hypothesized that the F1 male mice would spend less time than controls interacting with a novel partner. F1 males of the 40 and 400μg/kg did spend less time sitting side-by-side with their partner, but not less time interacting overall. These changes in social behavior could be explained by the impact DEHP and its metabolites have on neurodevelopment.

Particularly relevant to social behavior is the effect of DEHP on dopamine. Dopaminergic neuron numbers and tyrosine hydroxylase intensity in the midbrain is decreased in 6-week-old mice following 1mg/kg daily gestational and neonatal administration of DEHP (Tanida, Warita et al. 2009). Pertinent for sex differences and imperative to the role of steroid hormones on the developing brain, is the fact that DEHP has dose and sex dependent effects of aromatase in the hypothalamus (Andrade, Grande et al. 2006). DEHP also has effects on cell death, which is important to consider. *In vitro*, DEHP induces apoptosis on neurons in a neuroblastoma cell line via activation of Trim17 by PPARγ (Lin, Chen et al. 2011, Aung, Win-Shwe et al. 2014). It is not yet known how this relates to normal *in vivo* development, but PPARγ is an important part of the steroidogenesis pathway as well as highly active during gestational neurodevelopment (Lovekamp-Swan, Jetten et al. 2003, Heneka and Landreth 2007). Additionally, mouse neurons co-cultured with astrocytes have increased oxidative stress markers

and increased gliosis following exposure to biologically relevant DEHP levels (Wu, Li et al. 2014). Clearly, DEHP affects the brain in widespread ways that could have extensive effects on neurodevelopment and behavior, but the mechanisms for particular effects must be more thoroughly explored. Now that behavioral changes in each dose, sex, and generation have been identified, future studies will focus more directly on mRNA and protein changes in the brain on specific doses within this exposure model. This will provide insight into the impact on the human population especially during this critical time period.

Markedly, the F3 400µg/kg mice exhibited the opposite pattern in side-by-side sitting behavior of F1 mice, displaying more of this behavior and more social engagement throughout the task. The fact that the F1 data and the F3 data are not identical across generations clearly indicates the importance of direct versus epigenetic effects. It is not unusual for transgenerational results to differ from or be the opposite of effects in F1 offspring (Kubo, Arai et al. 2003, Gioiosa, Fissore et al. 2007, Wolstenholme, Edwards et al. 2012). However, the transgenerational data are slightly more difficult to interpret than the F1 results until the effects of DEHP on epigenetic mechanisms are better defined. Exposure to DEHP *in utero* produces an increase in global DNA methylation and increased expression of Dnmts in fetal testes (Wu, Zhu et al. 2010), which is likely to be representative of changes elsewhere in the body and brain. Studies of other endocrine disruptors have better characterized multigenerational and transgenerational mechanisms (Anway and Skinner 2006, Skinner, Manikkam et al. 2011, Wolstenholme, Rissman et al. 2011, Singh and Li 2012), but more research is necessary to determine mechanisms of heritable behavior effects as a result of DEHP exposure.

We recorded an increase in anxiety behavior in the 5 and 40µg/kg doses that is specific to F1 mice, and demonstrates the importance of dose selection on behavioral studies of environmental toxicants. This is the first example of anxiety behavior being affected by doses of DEHP this low. To date, there are several studies showing increased anxiety behavior in mice directly exposed to 10-540 mg/kg of DEHP (Carbone, Ponzo et al. 2013, Wang, Chen et al. 2014,

Park, Cheong et al. 2015, Xu, Yang et al. 2015). There is also unpublished elevated plus maze data from our lab showing no changes in F3 mice from 150mg/kg or 200 mg/kg doses. Taken together, these data imply that anxiety behavior is the result of direct DEHP exposure and not a result of transgenerational effects. DEHP metabolites interact with the HPA axis and the corticosterone pathway. DEHP metabolite MEHP regulates glucocorticoid metabolism by inhibiting 11beta-hydroxysteroid dehydrogenase 2, the enzyme that inactivates the stress hormone corticosterone (Hong, Li et al. 2009), presumably leading to increased corticosterone. Perhaps there is a compensatory mechanism in the F3 offspring, or this simply does not have long lasting effects.

Changes in anogenital distances offer potential clarity into multiple human studies showing decreased anogenital distance in males (Moore, Rudy et al. 2001, Swan, Main et al. 2005, Do, Stahlhut et al. 2012, Bornehag, Carlstedt et al. 2015). Animal models demonstrate that the reasons for this outcome are complicated as well as extremely dose and time dependent. Our data reveal an increase in female anogenital index in 5µg/kg mice from the F3 generation, but, somewhat surprisingly, we did not observe any statistically significant anogenital changes in males the day after birth from any doses in either generation. In the most comparable previous study in mice, anogenital index increases from controls were observed at 5µg/kg, and absent at 500µg/kg (Do, Stahlhut et al. 2012). However, the animals in this study were embryos dosed from gestational days 9-18 and collected at day 18. Furthermore, only male embryos next to one other male embryo *in utero* were selected (1M). Intrauterine position relative to other males directly and significantly affects the amount of testosterone each embryo is exposed to (vom Saal and Bronson 1980). We measured our mice following birth, so it was not possible to assess intrauterine position. Therefore, our data in comparison to previous mouse data only serves to further illustrate the extreme sensitivity of time and dose of testosterone during gestation. Human results may be due to continuous exposure throughout several generations. We did not observe

anogenital differences in males in our F3 mice, but it is possible that this would be a measurable effect following continuous DEHP throughout generations, or after measuring only 1M offspring.

Although the anogenital distance data in neonatal males did not indicate a difference in organizational testosterone, we did note lower testes weight in adult males from the F3 generation of our 5µg/kg dose. Several studies have shown testicular toxicity in rodent models both directly following DEHP exposure, as well as transgenerationally (Gray, Ostby et al. 2000, Borch, Metzdorff et al. 2006, Wu, Zhu et al. 2010, Doyle, Bowman et al. 2013, Ge, Han et al. 2015). Much of this research is focused on the antiandrogenic effects and uses doses much higher than the ones in this study. Unexpectedly, our data only denote lower testes weight in the lowest dose that we used and only in the F3 mice. As mentioned earlier, a previous study in mice does show an increase in global DNA methylation and increased expression of Dnmts in fetal testes in F3 mice, but this experiment used a dose of 500mg/kg (Wu, Zhu et al. 2010). Since our results are only in the 5µg/kg dose and not in the higher doses this likely represents another non-monotonic dose response curve where testicular toxicity exists at very high doses and very low doses. Interestingly, none of the doses showed differences in the F1 mice. Perhaps, the changes in methylation are more pervasive to this adult phenotype than direct interaction with the steroid hormone pathway. The effects on epigenetic mechanisms should be further researched at a variety of doses in order to better understand these data.

Conclusions:

In summary, we have shown that DEHP has dose, sex, and generation-dependent effects on social behavior, anxiety-like behavior, and reproductive development. The exact mechanism behind these results is unknown, but there is existing research on DEHP and its metabolites acting on several aspects on the steroidogenesis and neurodevelopment pathways. Future research should explore in more detail the effects of DEHP on gene and protein changes in the brain that are responsible for the diverse behavioral outcomes.

Chapter IV

Immune deficiency influences juvenile social behavior and maternal behavior

i. Introduction

Immune dysfunction is associated with learning impairments in mice (Brynskikh, Warren et al. 2008, Derecki, Cardani et al. 2010) and several psychiatric disorders in humans (Michel, Schmidt et al. 2012). Specifically, autism spectrum disorder (ASD) has been correlated with infection during pregnancy (Atladottir, Pedersen et al. 2009, Zerbo, Qian et al. 2013) and with familial autoimmune disease (Comi, Zimmerman et al. 1999, Atladottir, Pedersen et al. 2009, LY and Mostafa 2014). Although cognitive ability has been examined in mice that undergo developmental immune dysfunction, and social behavior has been evaluated following acute immune challenges, few studies have been conducted on social behavior and developmental immune dysfunction. Here, we use a mouse model with a mutation that prevents maturation of basic immune cells. Severe combined immunodeficiency (SCID) mice lack adaptive immunity caused by a spontaneous mutation in the *Prkdc* gene on chromosome 16, which impairs the recombination of antigen receptor genes and results in the interrupted development of B and T cells (Bosma, Custer et al. 1983, Bosma and Carroll 1991, Hsiao, McBride et al. 2012). Previous studies using SCID mice showed that adult males have impaired spatial learning, which is rescued with splenocyte, or purified T cell, replacement (Brynskikh, Warren et al. 2008, Derecki, Cardani et al. 2010). However, no other behaviors have been examined in this mouse model. In these studies, we evaluated two social behaviors in juvenile SCID mice. The juvenile time period is one time used for testing social behavior in mice (Pearson, Bettis et al. 2012), and childhood is a common time period for diagnosis of social disorders (Kogan, Blumberg et al. 2009). In these tests, first we observed behavioral differences between SCID and C57BL/6 (B6) mice, and next we asked whether splenocyte transfer to SCID pups could rescue social deficits.

Importantly, in all previous behavioral studies of behavior in SCID mice, SCID and B6 dams reared their own litters (Brynskikh, Warren et al. 2008, Derecki, Cardani et al. 2010). This design fails to take differences in maternal behavior into account, an important factor that influences offspring behaviors (Champagne, Francis et al. 2003, Schwendener, Meyer et al. 2009,

Zimmerberg and Sageser 2011). Therefore, in a third experiment, we observed maternal behavior in SCID versus B6 dams and found significant differences in time spent on the nest and nursing. In the last study, we bred B6 mice heterozygotic for the SCID gene mutation to generate litters composed of heterozygous, SCID, and wild-type (WT) pups. Our data demonstrate, for the first time, social deficits in male SCID mice, as well as differences in maternal behaviors between SCID and B6 dams.

ii. Materials and Methods

Animals

Breeding pairs of B6.CB17-*Prkdc*scid/SzJ (SCID, Stock#001913) were obtained from Jackson Laboratory (Bar Harbor, ME). Our colony C57BL/6J mice were originally purchased from Jackson Laboratory, bred and maintained in the University of Virginia School of Medicine, Jordan Hall Animal Facility. The University of Virginia Animal Care and Use Committee approved all procedures used and described here. All animals were maintained on a 12-hr light–dark cycle (lights off at 1300), and were provided food (Harlan Teklad diet #7912) and water ad libitum.

Experimental Designs

Experiment 1: Male and female neonates were left undisturbed in the home cage until weaning at postnatal Day 21 (PN21). A total of eight SCID and nine B6 males were tested (from three litters for each group) in the social recognition task first, followed one or more days later by the social preference task; this testing order remained the same throughout all experiments. The SCID males were tested as adults (between PN60 and PN90) in the olfaction test.

Experiment 2: Male pups received a single injection (intraperitoneal) of saline or purified splenocytes, on postnatal Day 7 (PN7; day of birth was counted as PN0).

Female littermates were not disturbed. A total of 12 SCID males (from six litters) were injected with saline, and 12 (from five litters) received splenocytes. Treatments were randomly assigned

by litter. We did not split treatments within litters because we were concerned that the stress produced by individually marking males within litters would be a confounding factor. An additional 14 B6 males (from four litters) received saline injections on PN7. Other than this brief intervention, animals were left undisturbed in the home cage.

Experiment 3: Ten SCID dams and 12 B6 dams were observed after parturition on PN1, PN6, PN7, and PN8. On PN7, pups received splenocyte or control saline injections (as in Experiment 2), and the B6 litters were either used for spleen donors or males received saline. Thus, on PN8, we observed four SCID dams with saline-injected pups, four with splenocyte replenished pups, and seven B6 dams with saline injected litters.

Experiment 4: Female SCID and male B6 mice were mated to produce heterozygous offspring to use for breeding pairs. Pairs heterozygous for the *Prkdc* mutation produced litters containing homozygous WT animals lacking the mutation (WT), mice that were heterozygous for the mutation (HET), and SCID mice with two copies of the mutated gene. The offspring from heterozygous pairs were genotyped using DNA from tail snips screened by polymerase chain reaction for *Prkdc* with the following primers: forward, 5'GGAAAAGAATTGGTATCCAC3'; reverse, 5'AGTTATAACAGCTGGGTTGGC3'. Only WT and SCID mice were tested. A total of six males (from four litters) that were homozygous for the SCID mutation, along with eight male (from six litters) WT littermates were tested. In all experiments, at weaning, male mice were group-housed with same-sex littermates (no more than five per cage). Between PN22 and PN27, the animals were tested for social preference and social recognition.

Juvenile Social Behavior (Experiments 1, 2, and 4)

Social preference: This test was performed as described previously (Cox, Gatewood et al. 2010). Briefly, 1 hr before testing, mice were moved (in their home cages) into the testing room. Mice were placed into a three-chambered box (76.2 cm x 26.67 cm x 17.78 cm) divided by black Plexiglas walls, and an outer wall was also black Plexiglas. Thus, the center section had dark walls on three sides, with two openings leading to the outer chambers. Each of the outer two

chambers contained an empty upside-down metal pencil holder (10.16-cm diam. x 13.97 cm), hereafter referred to as a "holding cell," with a round top and vertical bars (spaced 1 cm apart). Each mouse was habituated to the empty cage for 10 min, and then restricted to the center chamber while an adult B6 male mouse was placed randomly in one of the holding cells on one side of the test box. The doors were opened and the subject explored the entire box for 10 min. The test was conducted and recorded during the dark portion of the day–light cycle between 1300 and 1800 under red light. An observer blind to treatment group scored the amount of time the subject spent in each part of the test box, as well as time spent investigating the mouse or the empty holding cell. A preference score (the amount of time spent investigating the holding cell containing the male mouse—the amount of time spent investigating the empty holding cell) was calculated for each mouse.

Social recognition: This protocol was adapted from one described previously (Tejada and Rissman 2012, Wolstenholme, Goldsby et al. 2013). Mice were moved into the testing room and allowed to habituate in their home cages for at least 1 hr. The mice were then placed into a clean cage (the size of their home cages) containing a holding cell for 10 min, after which time an ovariectomized adult female was placed into the holding cell for 1 min. During each trial, the amount of time (in seconds) spent investigating was recorded. Here, we define investigation as nose contact between the subject and the other mouse or the bars of the holding cell. The 1-min trials were separated by 9 min, when the test male was alone in the test cage. Between trials, the ovariectomized females were single-housed in clean cages. This was repeated 8 times, and on the ninth trial a new stimulus animal (another ovariectomized adult female) was used in place of the familiar mouse. Testing was conducted during the light portion of the day/night cycle between 0800 and 1300, and behavior was scored in real time.

Olfaction Test (Experiment 1)

To habituate the mice to a novel food, Cocoa Puffs (General Mills, Inc.) were placed in their home cage in addition to their ad libitum food and water. Twenty-four hours later, all food was

removed from the food hopper and cage, and the mice were fasted overnight. The next morning, each mouse was placed alone in a clean cage with a Cocoa Puff hidden beneath the clean bedding. The time to find the hidden Cocoa Puff was recorded. This test was performed during the light portion of the day–light cycle between 0900 and 1200, and ability to locate the Cocoa Puff within 5 min was considered normal, as described previously (Yang and Crawley 2009).

Splenocyte Transfer (Experiment 2)

Splenocytes were collected from B6 pups (PN3-11), taken from litters whose pups were not used in these experiments. The splenocytes were collected and transferred to PN7 SCID pups. To collect the splenocytes, pups were wiped with ethanol and placed on ice for euthanasia, followed by rapid decapitation and spleen removal. The spleens were strained through a sterile cell strainer in phosphate buffered saline (PBS) containing 2% heat inactivated fetal bovine serum (FBS), and then transferred to a 15-mL tube with a transfer pipette. The cells were spun at 1100 rotations per minute for 8 min and the supernatant discarded. The resulting pellet was resuspended in ammonium chloride-potassium red blood cell lysis buffer and incubated on ice for 5 min. Then, the tube was filled with PBS containing 2% heat inactivated FBS and spun again at 1100 RPM for 8 min. The supernatant was discarded and the resulting pellet suspended in saline at a volume of 150µl/spleen collected. Each recipient received 150µl injected intraperitoneally, the equivalent of one donor spleen, using a sterile syringe and needle. Control-injected pups received 150 µl of sterile saline.

Maternal Behavior (Experiment 3)

First-time SCID and B6 dams (used to generate offspring for Experiment 2) were the subjects in this experiment. Dams were observed in their home cages for 30 min, once a day during the light (0100 to 1300), and a second time during the dark (1300 to 0100) on PN1 and PN6, and during the light on PN7 prior to injection. Thus, the preinjection observations were based on 2.5 hr of maternal behavior. On PN7, half of the B6 litters were euthanized for splenocyte collection. As

in Experiment 2, SCID mice received saline or splenocyte injections, and the remaining B6 litters received saline. We continued to observe maternal behavior during the dark portion of PN7 and twice during PN8 (light and dark) for an additional 1.5 hr of observations after the nests were disturbed and pups injected. We used scan-sampling methods to record maternal behaviors. We observed the nest every 15 s, recorded all behaviors, and noted if the dam was on or off of the nest. On the nest behaviors were licking and grooming pups, self-grooming, active nursing, passive nursing, nest building, and hovering. Behaviors off of the nest were eating and drinking, self-grooming, and digging and climbing. These methods were adapted from previous research (Chourbaji, Hoyer et al. 2011). Values were analyzed as proportions of total observations.

Statistical Analyses

The social recognition data were analyzed with repeated measures ANOVA. The factors used for the analysis were time spent investigating the stimulus animal and trial. Other data were analyzed using one-way ANOVAs. In all cases we used Fisher's Least Significant Difference posttests to detect pairwise differences.

iii. Results

Experiment 1: Social Behaviors Differ Between Juvenile Male SCID and B6 Mice

We measured time spent in all three sections of the three chambered test box and time investigating the holding cells (either empty or containing an adult male). When time investigating the empty holding cell was subtracted from time investigating a novel male mouse, SCID males had a larger preference for the novel mouse than did the B6 mice (**Figure 14a),** $F(1, 14) = 9.42$, $p = .009$. SCID mice also spent more time in the center chamber, $F(1, 14) = 5.58$, $p = .0344$, less time in the empty chamber, $F(1, 14) = 10.83$, $p = .006$, less time investigating the empty cell, $F(1, 14) = 5.20$, $p = .04$, and more time investigating the cell with the stimulus mouse, $F(1, 14) = 6.15$, $p = .026$, than B6 mice (**Table 7**).

In the social recognition task we found an interaction between trial and immune status for

Trials 1, 8, and 9 (**Figure 14b**), $F(2, 50) = 3.44$, $p = .045$. Planned comparisons indicated that all mice reduced investigation time between Trials 1 and 8 (B6, $p = .0001$;SCID, $p = .003$), indicating habituation but SCID mice failed to significantly increase investigation time between Trials 8 and 9 ($p = .099$), whereas mice did ($p = .0001$), suggesting an inability to dishabituate and recognize the novel female. All SCID male mice located the hidden food in the allotted time ($M = 97.63 = 19.8$ seconds), indicating that general olfaction in the SCID mouse is not impaired.

In sum, SCID males had a larger preference for social interactions with the stimulus mice and failed to show dishabituation in the social recognition experiment. Moreover, gross olfactory abilities were normal in SCID males. Thus, social behavior in two commonly used tests was affected by immune deficiency.

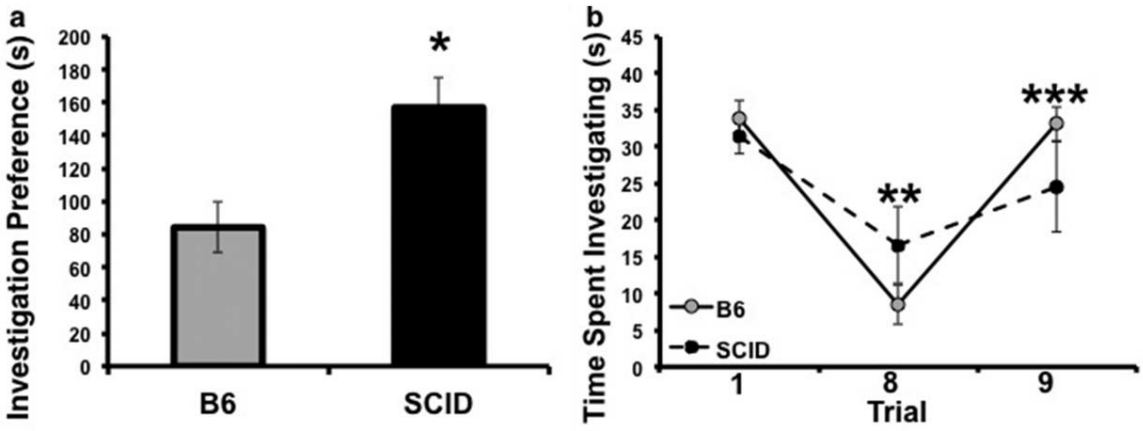

Figure 14: Means ± SEM SCID and B6 male mice examined in (a) social preference task, and (b) social recognition task. (a) SCID mice have higher preference scores for an adult male mouse than do B6 mice (* $p < .05$). (b) Both SCIDs and B6 male mice habituated to an ovariectomized mouse over eight trials (** $p < .05$), but only B6 mice dishabituated in response to a novel stimulus (*** $p < .05$). SCID, N = 8; B6, N = 9.

Social Preference Data for Experiments 1, 2, and 4: Time Spent in Each Chamber or Investigating a Holding Cell With or Without Another Mouse in it Presented as Mean ± SEM in Seconds

Experiment number	Group	Time in chamber with empty cell (s)	Time in center chamber (s)	Time in chamber with mouse (s)	Time spent investigating cell with mouse (s)	Time spent investigating empty cell (s)
1	B6	177.81 ± 17.88[a]	94.17 ± 13.02[a]	328.02 ± 14.21	124.87 ± 12.60[a]	40.63 ± 8.29[a]
1	SCID	95.61 ± 17.08	152.33 ± 24.15	352.06 ± 30.62	174.45 ± 16.86	17.44 ± 3.79
2	B6 + saline	168.9 ± 20.54	68.65 ± 13.81	362.59 ± 26.12	141.18 ± 14.07[a]	18.45 ± 5.25
2	SCID + saline	90.57 ± 16.76	62.08 ± 15.91	446.75 ± 27.42	245.42 ± 21.66	19.54 ± 5.28
2	SCID + splenocytes	194.97 ± 22.87	129.24 ± 23.07	275.14 ± 24.73	93.85 ± 17.01	50.92 ± 12.38
4	B6 males	253.74 ± 38.51	150.40 ± 17.30	196.14 ± 38.43	53.33 ± 12.39	16.77 ± 5.70
4	SCID males	253.79 ± 46.40	104.52 ± 15.21	241.80 ± 49.38	62.83 ± 15.39	26.22 ± 7.15

Table 7: [a]Significant differences between B6 groups from the same experiments and same column are $p<0.05$. ***

Experiment 2: Splenocyte Transfer Decreases Responses to a Novel Mouse and Ameliorates Dishabituation Deficit in Juvenile SCID Mice

SCID mice that received saline injections spent more time investigating the chamber with the stimulus mouse than B6 mice given saline and SCID mice that received splenocytes (see Table 7). The preference scores revealed that SCID given a splenocyte injection had a reduced preference for investigating a novel stimulus mouse than SCID mice that received saline (**Figure 15a**), $F(2,37) = 3.93$, $p = .029$.

In the social recognition task, an interaction between immune status and trial was found on Trials 1, 8, and 9 (**Figure 15b**), $F(4, 113) = 5.30$, $p = .0009$. Planned comparisons indicated that all three groups habituated to a novel stimulus (Trials 1 and 8, all groups, $p = .0001$). However, B6 and SCID mice that received splenocytes dishabituated (Trials 8 and 9, both groups, $p = .0001$), whereas SCID mice that received saline did not do so ($p = .162$; **Figure 15b**).

In sum, these experiments show that injection of splenocytes 2 weeks prior to testing suppressed social interactions in SCID mice. Yet, in the social recognition task, partial immune

restoration normalized dishabituation responses.

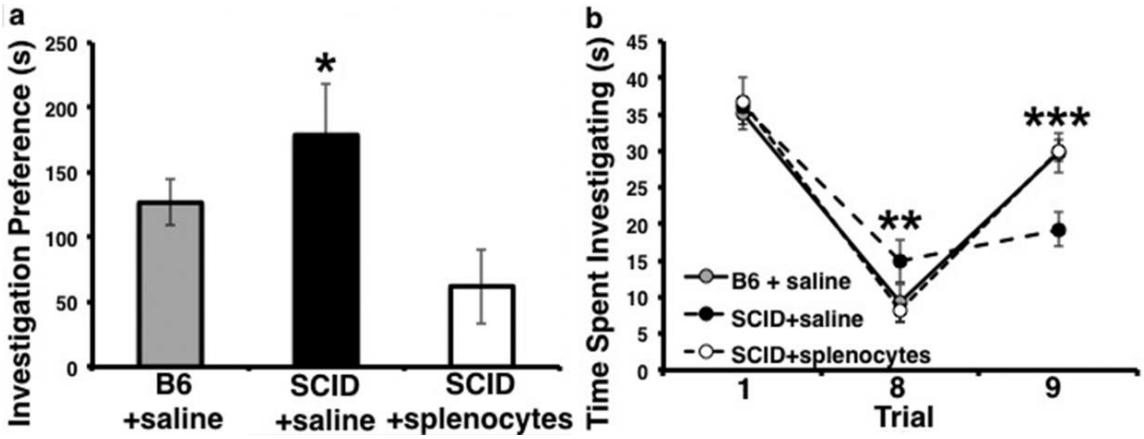

Figure 15: Means ± SEM SCID male mice with out without splenocytes replacement versus control B6 mice in (a) social preference task, and (b) social recognition task. (a) In the social preference task, SCID mice that received splenocytes have lower preference scores than SCID mice that receive saline (* $p < .05$). (b) SCID mice have impaired social recognition that is ameliorated with splenocyte transfer. All groups habituated to a stimulus mouse over eight trials (** $p < .001$). Only B6 and SCID mice with splenocyte transfer dishabituated on Trial 9 (*** $p < .001$). SCID + splenocytes, N = 12; SCID + saline, N = 12, B6, N = 14.

Experiment 3: SCID Dams Show Less Maternal Behavior Than B6 Dams

On P1, P6, and P7, SCID dams spent more time off of the nest (**Figure 16a**), $F(2, 100) = 4.96$, $p = .037$, less time on the nest (**Figure 16b**), $F(2, 100) = 4.97$, $p = .037$, and less time nursing (**Figure 16c**), $F(2, 100) = 9.24$, $p = .006$, their pups than B6 dams. Following nest disruption (a saline or splenocyte injection given to pups), SCID dams spent less time actively nursing their pups in an arched back posture (**Figure 16d**), $F(2,44) = 5.93$, $p = .016$, than B6 dams (**Figure 16d**). Several other behaviors were recorded, but none of these were different between the groups. Interestingly, these experiments show that maternal behaviors in SCID dams differ from controls in several dimensions. First, SCID dams spent less time nursing, and second, they were off the nest more frequently than B6 control dams.

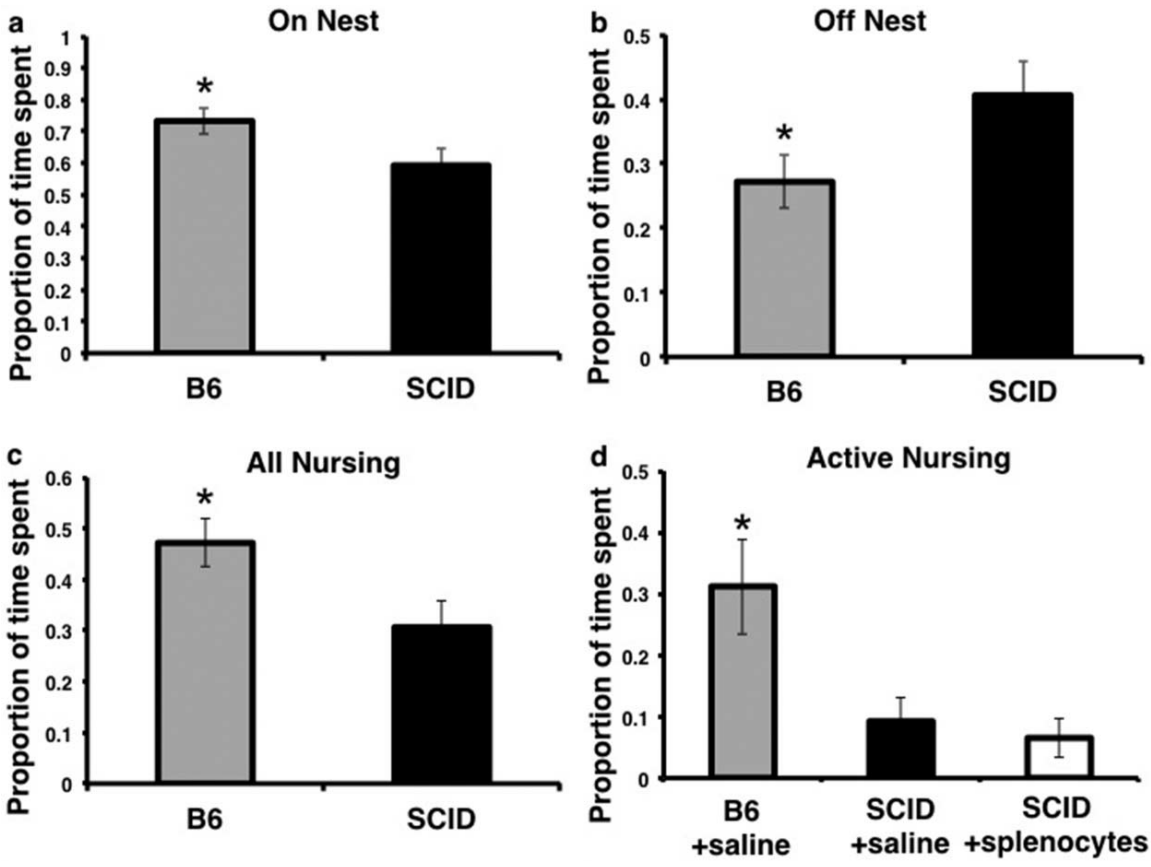

Figure 16: Means ± SEM Maternal behavior in SCID and B6 dams before pups are injected (a, b, c) and after injection (d). (a) On P1, P6, and P7, SCID dams spent less time on the nest than B6 dams (* $p < .05$), and (b) more time off of the nest (* $p < .05$) than B6 dams. (c) Prior to injection, SCID dams spend less time nursing than B6 dams (* $p < .05$). All measures were taken from SCID N = 12, and B6 N =12 on their nests. (d) Following a saline injection or splenocyte transfer to male pups on P7, SCID dams spend less time actively nursing their pups (on P7 and 8) than did B6 dams (* $p < .05$). SCID, N = 4, B6, N = 7, SCID + splenocytes, N = 4.

Experiment 4: SCID Mice Reared by Heterozygous Dams Display Social Recognition Deficits

In the social preference task, all mice spent more time investigating an adult male than an empty holding cell. No significant differences were observed for time spent in any chamber during the social preference test between genotypes, nor did social preference scores differ between the groups (**see Table 7**). In the social recognition task, an interaction between immune

status and trial was found on Trials 1, 8, and 9 (**Figure 17b**), $F(2,41) = 5.16$, $p = .014$, planned comparisons indicated that both groups habituate to a novel stimulus (B6, $p = .0001$; SCID, $p = .009$). However, only the WT B6 mice displayed dishabituation on Trial 9.

In summary, one important environmental factor, maternal behavior, was eliminated by use of heterozygous dams, and when this was done, SCID males had normal social investigation in a preference test. However, the SCID males still showed a failure to dishabituate in the social recognition task.

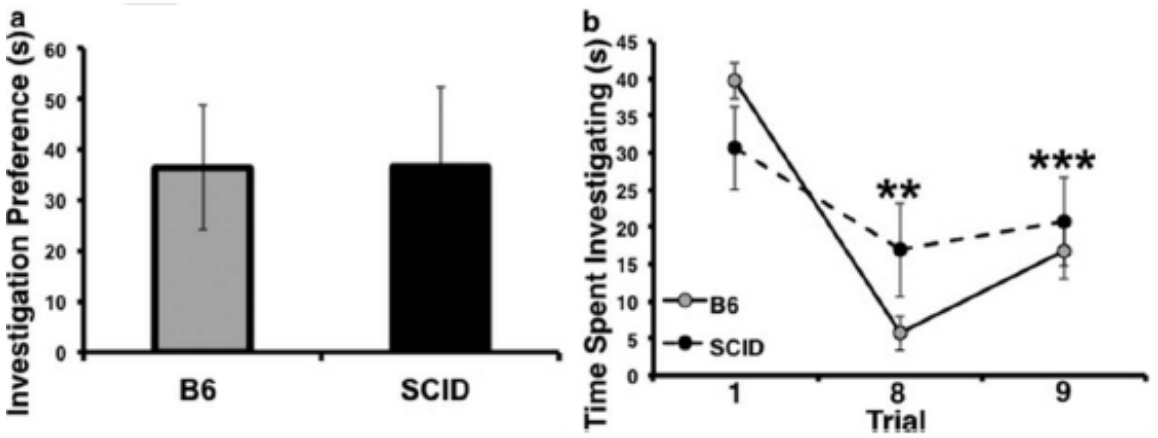

Figure 17: Means ± SEM SCID mice reared by heterozygous dams versus B6 mice reared by heterozygous dams in (a) a social preference task, and (b) a social recognition task. (a) In the social preference test, no significant differences in preference scores were found. (b) In the social recognition task, both SCID and B6 mice habituated to an ovariectomized mouse over eight trials (** $p < .05$), but only B6 mice dishabituate in response to a novel stimulus (*** $p < .05$).

iv. Discussion

Adult male SCID mice have spatial learning impairments that can be ameliorated by splenocyte replacement (Brynskikh, Warren et al. 2008, Derecki, Cardani et al. 2010). Based on these studies, the SCID has been suggested as a mutant model for autistic disorders (Derecki, Privman et al. 2010). Here, we examined two social behaviors in SCID mice, both of which are

relevant to autism. We found that SCID males had a larger preference for social interactions with the stimulus mice, and they failed to show dishabituation in the social recognition experiment. Moreover, gross olfactory abilities were normal in SCID males. Thus, social behaviors in two commonly used tests were affected by immune deficiency. Next, we asked whether providing SCID pups with splenocytes might restore some of these social behaviors. A single injection of splenocytes about two weeks prior to testing restored dishabituation, yet this same treatment resulted in reduced investigation levels to those lower than control B6 mice. One possible explanation for these results is that they are the result of a physiological immune response to foreign antigens from the splenocyte transfer, causing a reduction in activity. Alternatively the splenocyte dose may have been supraphysiological, producing a hyperactive immune response. However, the animals showed no sign of illness or physical impairment, and their general olfactory function was normal. Moreover, data from the social recognition test showed that the SCID splenocyte-replaced mice had normal habituation and dishabituation responses to restrained adult ovariectomized females. In addition to immune function differences, we asked whether maternal behaviors were different between SCID and WT dams. We noted that SCID dams spent more time off the nest and less time in the nest actively nursing than did B6 dams. Instead of doing a cross-fostering study in the final study, we presumed that heterozygous dams would display similar maternal behavior. Moreover, fostering itself can affect the behavior of pups tested as adults (Cox, So et al. 2013); thus, performing a complete cross-fostering study is a rather large undertaking. We produced pairs of heterozygotes and then assessed behavior in the male homozygous offspring (SCIDs and WTs). We found that the elevated social interactions in the preference test that were noted in the SCID males in Experiments 1 and 2 were not present when the offspring were produced by heterozygote pairs. Thus, rearing conditions and splenocyte restoration were both able to modulate behaviors in this task. On the other hand, the SCID males failed to dishabituate, an effect noted in all three studies. Yet splenocyte treatment in Experiment 2 was able to reverse this behavioral deficit.

Relevant to the role of immunity in these behavioral outcomes, anti-inflammatory cytokine release of Interleukin-4 in the meninges of the brain resulting from T cell activity can facilitate learning recovery in SCID mice (Derecki, Cardani et al. 2010). Therefore, it is reasonable to suggest that cytokine activity, or something downstream, is at least partially responsible for this shift in social learning. Although the SCID mice that received splenocytes experienced a healthy cell transfer, it is clear that there is a delicate balance of activity influencing social behavior.

The lack of dishabituation demonstrated by SCID mice when a novel female was presented during the final trial of the social recognition task suggests an inability to distinguish the novel mouse from the familiar. It has been shown previously that adult mice lacking the recombination-activating gene RAG1 are also deficient in social recognition tasks (McGowan, Hope et al. 2011). RAG1 (along with RAG2) mediates variable (diversity) joining recombination, necessary for the maturation of B and T cells (McGowan, Hope et al. 2011). This variable joining recombination is the same process that is disrupted by the *Prkdc* gene in SCID mice, causing their lack of T and B cells (Bosma, Custer et al. 1983, Bosma and Carroll 1991). These data indicate that this process and these cells are important for social recognition in adults as well as in juvenile mice. However, SCID mice that received splenocytes were able to dishabituate in the social recognition paradigm, indicating that this partial immune restoration restores the ability to recognize a novel social stimulus. We speculate that this same procedure would restore dishabituation in RAG mutants also.

Some mouse models for autism exhibit social behavior deficits and also have deficiencies in immune function. For example, there is evidence for immune system disruption in the ASD mouse model BTBR T = tf/J (BTBR). The BTBR strain has higher levels of serum IgG and IgE, as well as elevated expression of proinflammatory cytokines IL-33, IL18, and IL1= in the brain compared with B6 control mice (Heo, Zhang et al. 2011). Furthermore, previous studies have shown decreased social behavior of offspring whose dams had infections during gestation; this is

likely linked to increased cytokine activity (Malkova, Yu et al. 2012). Additionally, reversal of social behavior deficits in a maternal immune activation (MIA) model of poly (I:C) injection is noted after MIA offspring received bone marrow transplantation from immunologically healthy mice, as this procedure rescues a proinflammatory phenotype (Hsiao, McBride et al. 2012). Our data add to the literature supporting the idea that normal social recognition and cognition are dependent, at least partially, upon normal immune function.

The mechanisms responsible for the behavioral differences between SCID and B6 males are unclear, but the current data does supply us with the necessary information to pursue this question in future studies. Importantly, the social behavior tasks we used are considered to be amygdala-dependent in mice (Ferguson, Aldag et al. 2001, Keverne and Curley 2004), so this region is likely to be directly affected, as are genes known to be involved in these social behaviors, such as oxytocin and vasopressin (Keverne and Curley 2004). In previous research regarding the immune system and social behavior, rats were exposed *in utero* to an immune challenge, most commonly lipopolysaccharide (LPS). LPS exposed male, but not female, rats displayed less juvenile play behavior than controls, and this behavioral change was correlated with reduced expression of vasopressin mRNA (Taylor, Veenema et al. 2012). However, if the same immune-driven learning mechanisms are acting here, it is reasonable to hypothesize that spatial memory, and therefore hippocampal activity, may be critical as well. Cytokine release from T cells is an important element in the regulatory mechanisms of spatiocognitive deficits in SCID mice (Derecki, Cardani et al. 2010), and may also influence the social cognition deficits seen in our study.

Our initial experiments, and all previous work on behavior in SCID mice, used paradigms without any consideration of potential effects of the dams' maternal behavior and/or heterogeneity of littermates. Moreover, no assessment of maternal behavior has been conducted with these mice. This information is of value and arguably necessary for any future research using SCID mice. Maternal care in the form of licking, grooming, and nursing behavior has been shown

in rats to affect the maternal behavior of offspring, in which pups exhibit the behavior that their mothers provide (Champagne, Francis et al. 2003, Romeo, Mueller et al. 2003, Bailoo, Jordan et al. 2014)

Furthermore, maternal separation alters play behavior (Zimmerberg and Sageser 2011), and maternal care and experience in general affect a variety of behaviors and changes in the brain in juvenile and adults offspring of mice and rats (Ressler and Anderson 1973, Suomi 1997, Mousseau and Fox 1998, Qvarnstrom and Price 2001, Weaver, Meaney et al. 2006, Smit-Rigter, Champagne et al. 2009). Here, we show for the first time that SCID dams spend less time on the nest and have less attentive nursing behavior than B6 dams. This has important implications for any future studies using these mice as a behavioral model.

In Experiment 3, we quantified maternal care in WT and mutant SCID dams. Results of Experiment 3 indicated that SCID mice display differences in quality and quantity of maternal behavior compared with B6 mice; following this observation, we conducted another experiment using heterozygous pairs to create mixed litters. In our paradigm, all mice received care from heterozygous dams. We observed that the social preference behavior of these offspring was similar in the SCIDs and B6 mice. On the other hand, social recognition behavior remained deficit in the SCID male mice compared with B6 mice, as previously observed. In Experiment 2, the phenotype was modified by splenocyte transfer. Thus, this behavior may not be regulated by effects of maternal care, but could be more directly influenced by peripheral immune status.

The data presented could provide insight into social behaviors in humans that are at risk in individuals with immune deficiencies. Understanding subtle differences in juvenile social behavior should enhance our ability to diagnose early childhood diseases such as ASD. In certain populations, children with ASD have been shown to have immune deficiencies (Jyonouchi, Geng et al. 2008). Specifically, cytokine responses can be dysregulated, and may be attributed to the child's environment (Goines and Ashwood 2012), which is why the interaction between early life stresses and other environmental disruptions with immune dysfunction deserve more

investigation (van Gent, Heijnen et al. 1997, Michel, Schmidt et al. 2012). This is further illustrated in animal studies using human antibodies. When IgG from human mothers of children with autism are administered to pregnant mice, the offspring show increased anxiety and fear responses (Singer, Morris et al. 2009). Taken together, our current results show that immune deficiency can modify social behavior in juveniles, and that normal splenocytes, given prior to weaning, modify these differences. However, maternal behavior is different between SCID and B6 dams both prior to and after nest disruptions, and this may well affect the behavior of the offspring as well. The SCID mouse may be a useful model for investigating immune function in social behaviors and uncovering the mechanisms underlying immune actions on behavior.

Chapter V

Conclusions, Implications, and Future Directions

i. DEHP and Behavior, Hormones, and Stress

In this dissertation, I primarily examined the effects of the endocrine disrupting chemical DEHP on behavior, stress response, and reproductive development. EDCs are ubiquitous in the environment, and a range of compounds is detectable in humans (Crinnion 2010). DEHP is an EDC that is widespread (McKee, Butala et al. 2004, Halden 2010) and interferes with several points on the steroid hormone pathway (Hannon and Flaws 2015). However, the doses, timeframe of exposure, and mechanisms responsible for effects in humans are not well understood. My studies help provide guidance towards mechanisms that are relevant for changes in human social behavior and reproductive dysfunction. I have shown here for the first time, that DEHP has transgenerational actions in both sexes on the neuroendocrine axes and behaviors. I have also revealed for the first time that mice given human relevant doses demonstrate changes in behavior following *in utero* exposure to DEHP and several generations removed. Moreover, I observed effects on anogenital distance, body weight, and testes weight some of which suggest that the transgenerational target of DEHP is the HPA axis.

The most significant portion of my work may be analyzing the effect of DEHP on social behavior. The data linking levels of DEHP and its metabolites to altered social behavior and autism diagnosis in children strongly suggest that DEHP affects neurodevelopment (Swan, Liu et al. 2010, Testa, Nuti et al. 2012, Braun, Sathyanarayana et al. 2013, Park, Cheong et al. 2015, Park, Lee et al. 2015). In humans, however, it is impossible to determine exact doses, examine impact on the brain, and to differentiate between direct and transgenerational effects. This data is complex and animal research is crucial to its interpretation. To date, only one other animal study has been conducted looking at the effects of DEHP on social behavior. This study found that administration of 30mg/kg of DEHP to dams during gestation resulted in decreased social interaction in the offspring (Lee, Chiang et al. 2015). While this research is important, it only reports on one dose, and does not differentiate between the effects on males and females. I have

addressed these issues by conducting studies on social and anxiety behavior, stress response, and reproductive system development in several doses *in utero* and during lactation, which mimics human conditions. In the previous chapters, I have shown that a wide range of doses, including low doses, have direct and transgenerational effects on males and females. Specifically, DEHP doses ranging from 5-400μg/kg *in utero* and during lactation have effects on social interaction and anxiety behavior in juveniles and there are different, sometimes opposite, changes in F3 animals descendent from exposure. Additionally, doses of 150mg/kg and 200 mg/kg administered during embryonic days 7-14 have transgenerational effects on social behavior, stress response, and reproductive development, further characterizing this gestational window as a critical period for heritable changes.

I hypothesize that exposure to DEHP *in utero* has an immediate effect on neurodevelopment, at least partially through interaction with steroid hormone receptors in the developing brain. High doses (antiandrogenic due to decreased testosterone following exposure) and low doses (androgenic due to increased testosterone following exposure) are both present in human environments. All of these alterations on fetuses and developing neonates, as well as their potential offspring, create a cyclical and compounding effect of direct and transgenerational exposure; this is what truly creates the effects that we see in human populations today. A few key changes in testosterone, estradiol, aromatase and corticosterone enzymes are the most well characterized outcomes that are likely to affect people (**Figure 18**). Furthermore, several studies have shown that DEHP exposure causes neuronal death (Lin, Chen et al. 2011, Guida, Laudati et al. 2014, Yang, Zhang et al. 2014), but all of these studies have been done *in vitro* with the exception of one *in vivo* result of reduced overall brain weight (Tanida, Warita et al. 2009). Other more extensive *in vivo* research shows that prenatal doses of DEHP at 10, 50 and 200mg/kg lower hippocampal ERβ protein females and AR protein in males. Importantly, the AR differences are no longer visible at 12 weeks of age (Xu, Yang et al. 2015). This suggests that gene expression

effects of *in utero* exposure are transient, and, given the significance of hormone receptors in these early stages of development, it is important to characterize them. Therefore, experiments at the mRNA level close to and just prior to the time of birth are very relevant. Currently, studies are being conducted to evaluate corresponding gene transcription changes in placentas and embryonic brains. I hypothesize that females receiving low doses have masculinized hormone receptor patterns and that the reverse is true in males receiving high doses. The results from this gene expression analysis will provide further insight into the activity that is regulating the behavioral changes described previously. Additionally, the results from the hippocampus are in high, antiandrogenic doses, so I predict that an increase in AR in females may result from the lower, androgenic doses used in my experiments. My work has highlighted changes in social behavior that provide a strong foundation of relevant doses for juvenile effects, creating a basis for investigating genes of hormone receptors and related to social behavior.

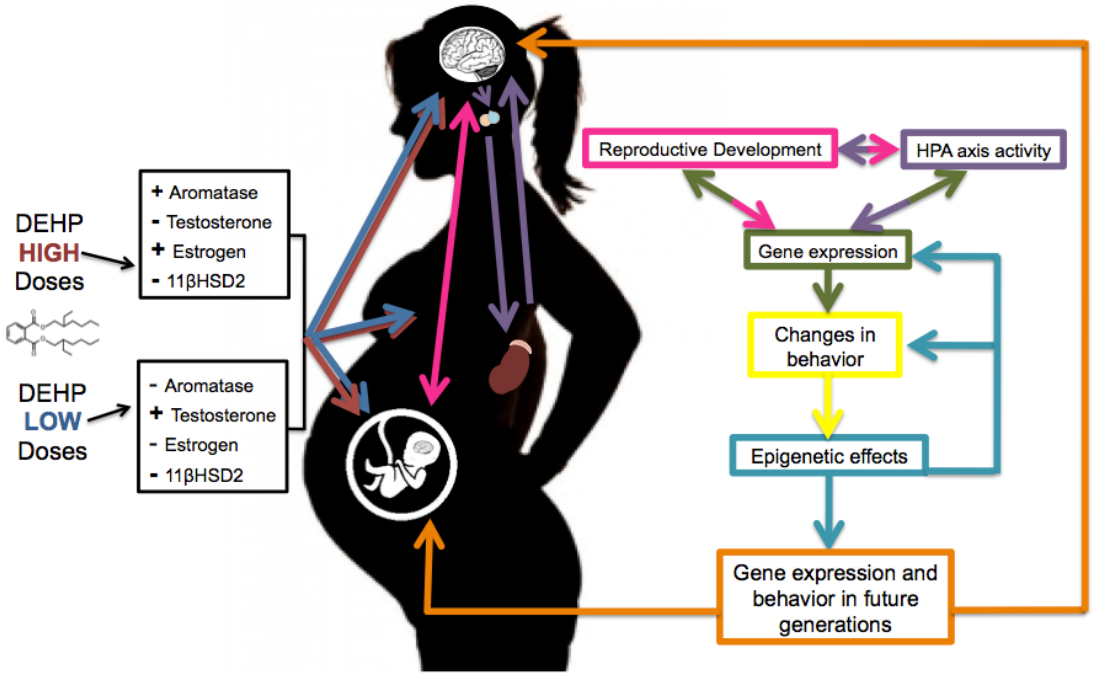

Figure 18: DEHP has complex and dose dependent effects directly on individuals, as well as during gestation at high doses and low doses. Altered hormone levels change reproductive development in the gonads and brain, HPA axis activity and stress/anxiety responses, as well as other genetic and epigenetic mechanisms. All of these changes on fetuses, as well as their potential offspring create a cyclical effect of compounding direct and transgenerational exposure, creating the effects that we see in humans today.

The difference that I have observed in social behavior between the F1 and F3 animals is particularly fascinating. This same effect has been recorded previously in our lab, with a different EDC, BPA (Wolstenholme, Edwards et al. 2012), but this is the first indication of DEHP having a similar effect. The fact that this has now been observed in multiple EDCs suggests a common method that makes direct effects different than those seen across generations. Seeing as the social behavior phenotype switches from one generation to the next, I assume that different mechanisms than in the F1 animals are responsible for changes in F3 animals. Based on previous research on DEHP and other EDCs, I believe that an epigenetic effect creates a permanent change in the F1s that persists following DEHP exposure that is responsible for the behavior differences in the F3 animals. While there are immediate effects via hormone receptors in F0 and F1 animals, it is likely that these specific actions do not continue in subsequent generations. Little is known about the epigenetics of DEHP in the brain, but it does alter methylation in the testes (Wu, Zhu et al. 2010). Moreover, there are transgenerational effects of disturbed germ cell association, lower sperm counts and decreased motility in F3 animals (Doyle, Bowman et al. 2013), and changes in methylation patterns in maternally and paternally imprinted genes in F1 and F2 oocytes (Li, Zhang et al. 2014). Taken together with my transgenerational data on behavior, this suggests that similar mechanisms are acting in the brain. I hypothesize that the changes in imprinted genes in embryos affect gene expression in the brain and that the testes data is also representative of long-term neurological changes. Furthermore, a previous study showed that mice exposed to 200mg/kg of DEHP prenatally and during weaning display increased grooming behavior (Dai, Yang et al. 2015). My results in Chapter II in male mice exposed to 200mg/kg between days 7-14 of gestation show decreased grooming, a possible additional indicator of opposite behaviors in F1 and F3 animals. The mice in the study showing increased grooming also had decreased NMDA receptor protein in the hippocampus, making this another target for further investigation.

The doses that I used in Chapter II are comparable to those used in the studies that found transgenerational changes in the testes. In the 150mg/kg dose, males had smaller seminal vesicles

and increased anogenital distances. In addition, my dose response study shows that males that received a 5ug/kg dose had lowered testes weights, signifying that much lower doses have heritable effects on methylation in the testes as well. In humans, studies correlate levels of DEHP metabolites with decreased anogenital distance and elevated risk of hypospadias in newborn males (Jensen, Anand-Ivell et al. 2015, Swan, Sathyanarayana et al. 2015). Even as more studies develop, some of them at lower doses, the vast majority of reproductive research is done with very high amounts of DEHP. This research is relevant, especially since the level of exposure in infants in intensive care units and individuals who work in certain industries is higher than previously thought, but it is necessary to study a wider range of doses than has been researched thus far. DEHP has a non-monotonic dose response and the mechanistic differences at hormone receptors and in different parts of the neurosteroid pathway at different doses remains unclear. My results are relevant to the human data because they show dose sensitivity and different effects in F1s and F3s. This is representative of the complex differences seen in people. It also reveals that the correlations in people are complicated by continued exposure, and may be the outcome of transgenerational changes and not direct effects. Future research with sustained exposure across several generations would help to parse out these differences.

The information from both Chapters II and III on stress response and anxiety behavior provides novel insight and brings further important questions to light. Although it is already known that the DEHP metabolite MEHP inhibits inhibition of corticosterone via 11βHSD2 (Hong, Li et al. 2009, Zhao, Chu et al. 2010), there were no published studies investigating transgenerational anxiety behavior at any doses prior to the research presented in this dissertation. While I did conduct anxiety tests at several doses, the only significant results showing an increase in anxiety behavior were in the F1 animals of the 5μg/kg and 40μg/kg doses. This leads me to believe that effects on anxiety behavior do not have epigenetic effects and that these results are the product of direct contact with DEHP. Presumably, the actions of MEHP on 11βHSD2 do not

create a persistent epigenetic mark and only affect anxiety in F1 animals at specific doses. Given my research and the other existing studies on anxiety, it seems that this is specific to very low and high doses. Other published studies show increased anxiety at doses in a range of 10-200mg/kg (Carbone, Ponzo et al. 2013, Wang, Chen et al. 2014, Park, Cheong et al. 2015, Xu, Yang et al. 2015). I did not observe an anxiety phenotype at the 200 mg/kg dose, but the mice that I tested were all from the F3 generation, further illustrating the importance of direct versus heritable outcomes. I did, however, observe lower serum corticosterone in F3 females at the 150mg/kg dose. The fact that this does not correspond to anxiety behavior indicates that it is specific to acute stress response and not a generalized anxiety phenotype. So far, I have only observed a transgenerational change in corticosterone in this very high dose, suggesting that heritable changes in this pathway result from large amounts of DEHP. Information on anxiety related to DEHP in humans is lacking. Perhaps publishing research that shows transgenerational results and low-dose results will lead to more extensive investigation of anxiety and stress related to DEHP exposure in humans. This would have direct relevance to several of the other behavioral changes and neurobehavioral disorders already studied in people.

In conclusion, DEHP has wide reaching effects on development and behavior that spans generations. Although it is obvious that the effects of DEHP on the steroid hormone pathway affect the reproductive system, detailed studies related to the brain and behavior are lacking. In order to better understand these data and the mechanisms behind them, *in vivo* and *in vitro* models that allow a closer look at molecular mechanisms are needed. The information in these chapters provides novel details on outcomes of specific doses, relevant windows of exposure, and differences between males and females. Future research will elucidate more in depth information about the mechanisms responsible for the differences that I have observed.

ii. The Immune System and Juvenile and Maternal Behavior

I also conducted studies the effects of the immune system on juvenile social behavior and maternal behavior. These experiments illustrate a strong developmental effect of

immune deficiency on social behavior. I conducted the studies using the genetic SCID model. These experiments are essential to more clearly define the differences between developmental and acute immune deficiency. Previously, splenocytes in SCID mice have been shown to rescue a learning deficiency in SCIDs (Brynskikh, Warren et al. 2008). In these studies, I evaluated two social behaviors in juvenile SCID mice. The juvenile time period is one time used for testing social behavior in mice, and childhood is a common time period for diagnosis of social disorders. First, I observed behavioral differences between SCID and C57BL/6 (B6) mice, and next asked whether splenocyte transfer to SCID pups could rescue social deficits. My studies showed for the first time that SCID mice have impaired social recognition that is rescued by injection of healthy splenocytes. I also discovered that SCID dams have impaired maternal behavior that affects certain juvenile behaviors. This information can be used to better evaluate complex effects of autoimmune disorders that affect the environment *in utero* and have long lasting effects on behavior of offspring.

Maternal care is accepted as a factor that changes the behavior of offspring and has long term and measurable effects (Weaver, Cervoni et al. 2004). Little is known, however, about the effect of the immune system on maternal behavior. The studies that do exist primarily use acute exposure to lipopolysaccharide (Hood, Dreschel et al. 2003, Penteado, Teodorov et al. 2014) and maternal behavior has never been evaluated in the SCID model. My research shows a reduction in maternal behavior of SCID dams compared to controls. Furthermore, this deficit plays a role in altered juvenile social preference behavior, which is rescued with care by the same dams between control and SCID litters. This information is especially significant for anyone studying behavior in immune models.

iii. General Conclusions

In this dissertation, I assert that the environmental endocrine disrupting chemical DEHP and the immune system play important roles in neurodevelopment and behavior and further

defined what these roles are. Here, I show in mice that lower doses than ever before studied, in the range of average human consumption, alter behavior, and have heritable effects in generations removed from exposure. Higher doses administered during embryonic days 7-14 have transgenerational effects on social behavior, stress response, and reproductive development, further characterizing this gestational window as a critical period of development and exposure to EDCs. This research has implications for human reproductive development and neurobehavioral disorders.

During time *in utero*, rearing, and adulthood the immune system plays a very important role in behavior (Brynskikh, Warren et al. 2008, Derecki, Cardani et al. 2010, Ruff, Nelson et al. 2012, Hsiao 2013, Foley, Macfabe et al. 2014). I have shown in a SCID mouse model that not only does developmental and genetic immune deficiency mediate juvenile social behavior, it also has profound effects on maternal behavior. The SCID model has never been used to test social or maternal behavior before and provides unique insight into this particular genetic mechanism. This data expands on the hypothesis that immune system activity influences social behavior and is relevant to neurobehavioral disorders in humans. The work in this dissertation is novel to several fields and holds much potential for future exploration.

Appendix A

Original data from 5µg/kg DEHP pilot study

Before the extensive studies of DEHP that were described in previous chapters, a low dose pilot study was conducted. Three main findings were discovered illustrating the potential for further research on the subject. Although the results are not completely in line with the 5μg/kg dose from the dose response study in Chapter III, the paradigm was slightly different. In this pilot research, dams consumed a phytoestrogen free diet; as opposed to the low phytoestrogen diet described in chapters II and III. Furthermore, pups were only exposed to DEHP during gestation, and not lactation. For the more extensive studies that followed we chose to give dams DEHP during gestation and lactation to better imitate human exposure. We expected this dose to be androgenic, as it falls on the portion of the curve that resulted in increased testosterone in previous studies (Do, Stahlhut et al. 2012).

The first finding was that pups exposed to DEHP *in utero* have louder vocalizations (**Figure 19**). Pup vocalizations are a common measurement of neonatal behavior and can be used to assess neurodevelopment (Scattoni, Crawley et al. 2009). Call volume is difficult to interpret, and simply led me to the conclusion that vocalizations in DEHP exposed animals are different than controls, and that more complex studies would yield more conclusive results. Furthermore, it led me to question maternal behavior in DEHP dams, as calling is also a response to mother-pup interaction. I assessed this in Chapter III and found no significant differences. Therefore, the differences in pup calling are almost certainly due to neurodevelopmental alterations by DEHP.

It was also discovered that adult females spent more time in the corner of the open field that control females (**Figure 20**). Time spent in the corners in the open field is indicative of anxiety (Walsh and Cummins 1976). However, we also conducted testing in the elevated plus maze, the most notable anxiety behavior test, and saw no differences. Therefore, it is difficult to draw conclusions from these results. Perhaps the visibility of the entire field decreases desire to explore, whereas in the elevated plus maze the closed arms are not visible from the open arms and vice versa. If DEHP exposed mice exhibited difficulty in a place learning paradigm, which would provide more insight into these results.

Interestingly, I also observed a sex and DEHP exposure specific deficit in social recognition (**Figure 21**). Social recognition is an established behavior with defined neural pathways, and an important representation of normal social development and social learning (Ferguson, Young et al. 2002). Because the results in this test indicate that DEHP exposed females behave like control males and unlike control females, I concluded that my original hypothesis about testosterone increase in this dose was correct. These animals were juveniles, having no endogenous circulating hormones of their own; thus, this masculinization was the result of organizational testosterone during gestation.

In conclusion, this pilot provided enough information to continue testing the effects of DEHP on behavior in mice, and to expand the paradigm. This low dose is the lowest dose used in the more extensive dose response experiments, and the pilot results indicated that using very low human relevant doses would produce measurable results.

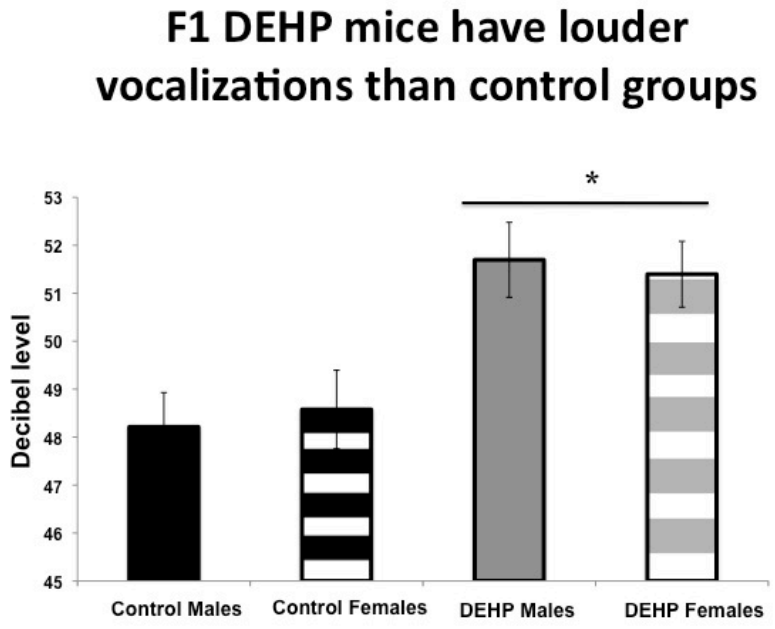

Figure 19: Nine day old DEHP exposed mice of both sexes have louder vocalizations that control mice.

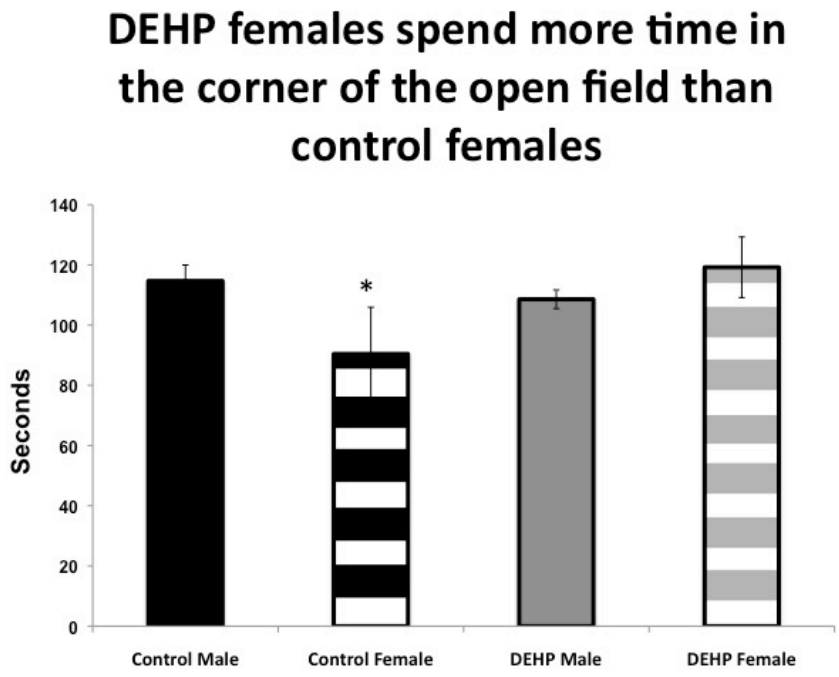

Figure 20: Adult female DEHP exposed mice spend a longer amount of time than control females in the corner of a square open field during a 10-minute test.

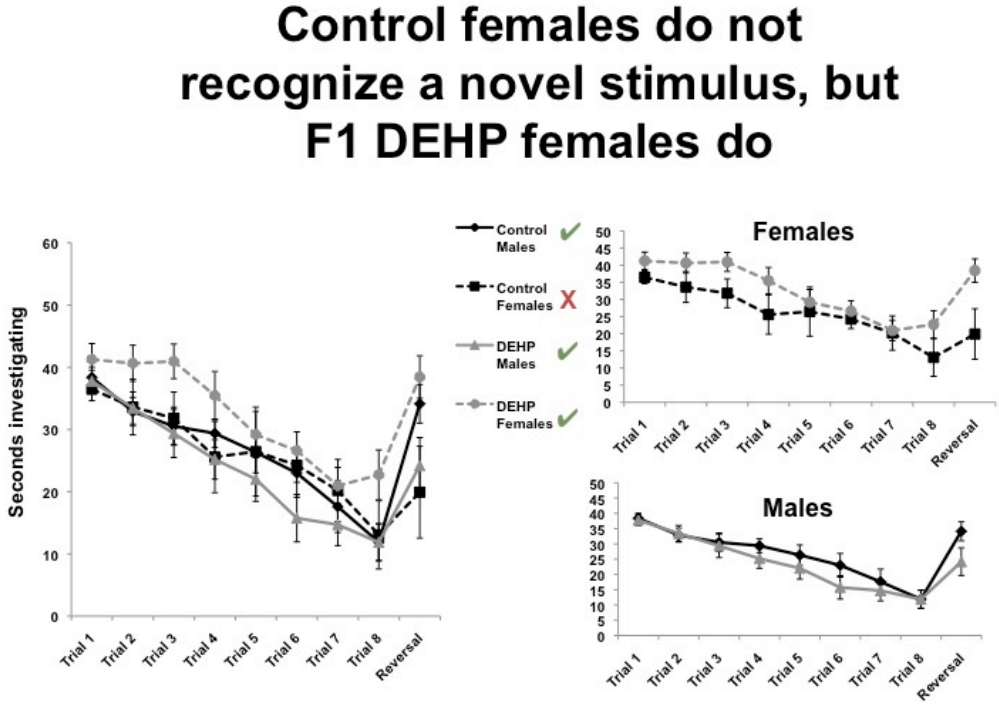

Figure 21: Juvenile male mice have social recognition of a novel adult ovariexctomized female, but control females do not. DEHP females display masculinized behavior, exhibiting normal habituation and dishabituation to a novel stimulus.

Appendix B

Neural growth hormone: regional regulation by estradiol and/or sex chromosome complement in male and female mice

i. Introduction

Growth hormone (GH) is synthesized in the pituitary and the brain (Kopchick and Andry 2000, Harvey 2010). Notably, like other anterior pituitary hormones, the secretion of GH is pulsatile and controlled by metabolic and neuroendocrine mechanisms (Painson and Tannenbaum 1991, Shah, Evans et al. 1999, Veldhuis, Anderson et al. 2004). There are long-established and well-documented sex differences in the frequency and amplitude of these GH pulses beginning during the peripubertal period in many mammalian species, including humans (Muller 1987). GH is also produced in the brain, where its distribution is extensive. GH receptors are present throughout the brain, and animal and human studies have implicated central GH signaling in neural functions (Harvey and Hull 2003). For example, GH levels influence or are correlated with cognitive performance (Falleti, Maruff et al. 2006) and enhance excitatory hippocampal synaptic transmission (Mahmoud and Grover 2006). GH also has neuroprotective effects, protecting from age-related hippocampal deficits in plasticity and learning (Ramsey, Weiner et al. 2004) and against cerebellar damage following ischemic injury (Alba-Betancourt, Luna-Acosta et al. 2013). Additionally, GH has been broadly implicated in aging and neurogenesis, as neural precursor cells in the subventricular zone proliferate in response to central GH infusion (Blackmore, Reynolds et al. 2012). Feeding behavior has also been linked to GH (Bohlooly, Olsson et al. 2005), and GH expression, in the hypothalamus specifically, is associated with sexually dimorphic body weight differences (Bonthuis and Rissman 2013).

Regulation of GH differs by brain region. For example, in the hippocampus, *Gh* mRNA levels are lower prior to puberty and increased in post pubertal rats (Donahue, Kosik et al. 2006). Also, *Gh* mRNA and protein expression are noted in many brain regions and known to be sexually dimorphic (females have higher mRNA expression and protein levels than males) in the hippocampus, preoptic area of the hypothalamus (mPOA), and arcuate nucleus in mice (Addison and Rissman 2012). While sex differences in GH levels and release have been documented, the mechanisms that regulate these sex differences are not well explored.

Estradiol modulates GH protein and *Gh* mRNA expression in the rat hippocampus. In females, GH protein levels in the hippocampus are highest during estrus, when estradiol levels are elevated. In addition, ovariectomized rats express low levels of *Gh* mRNA and protein which increase following estradiol treatment (Donahue, Kosik et al. 2006). In mice, the arcuate nucleus and the mPOA express sexually dimorphic *Gh* mRNA with females having higher levels than males. After gonadectomy, *Gh* mRNA decreases in females but not in males. Interestingly, castration actually increases *Gh* expression in the mPOA. Neurons containing GH protein also contain estrogen receptor alpha, and the anti-estrogen tamoxifen blocks the effects of estradiol on GH in females (Addison and Rissman 2012). Thus, estradiol may regulate GH in females but not in males. In addition, the numbers of X chromosomes have a direct effect on *Gh* mRNA in the mouse mPOA (animals with two X chromosomes have higher levels than those with one X), and two genes on the X chromosome known to escape X inactivation are correlated with *Gh* expression in this region (Bonthuis and Rissman 2013).

Taken together, existing data suggest that both estradiol and sex chromosome complement have actions on *Gh* mRNA and protein in a region-specific manner in the brain. Given the broad implications and regional specificity of GH, its regulation is important to assess. In this report, we tested these two factors simultaneously in three diverse brain regions: the hypothalamus, hippocampus, and cerebellum. We used the four core genotypes (FCG) mice, which have been previously utilized to compare sex chromosome versus gonadal sex as sources of sex differences (De Vries, Rissman et al. 2002, Arnold and Chen 2009). In the FCG mice, gonadal sex is unlinked from the sex chromosomes by deletion of the sex-determining region on the Y chromosome (*Sry*) and transgenic insertion of *Sry* on an autosome (Cox, Bonthuis et al. 2014). The FCG cross produces four types of offspring: normal females with ovaries and XX chromosome genotype (XXF), females with ovaries and XY genotype (XYF), males with testes and XY genotype (XYM), and males with testes and XX genotype (XX males). To manipulate estradiol, adult gonadectomized mice were treated with chronic implants containing estradiol or

no hormone. We report that estradiol treatment increased *Gh* mRNA significantly in the hippocampus and cerebellum. Sex chromosome complement was a factor only in the hypothalamus where XY females had the highest levels of GH protein and mRNA. Because the sex chromosome complement effect was in the opposite direction from our past reports in wild type and another sex chromosome mutant mouse model, we quantified GH protein in the arcuate nucleus. We replicated our previous finding that XX mice have more GH than XY mice in the arcuate, regardless of gonadal sex.

ii. Materials and Methods

Animals

All procedures were approved by and conducted in accordance with the University of Virginia Animal Care and Use Committee guidelines. The mice used for all experiments were the FCG mice on a C57BL/6J background. The FCG mice are XX females, XY females, XX males, and XY males (De Vries, Rissman et al. 2002). Mice were maintained on a 12:12 light cycle (lights on at 1:00 pm). Animals had access to water and food (# 7912 from Harlan Teklad, Madison, WI) *ad libitum*.

Gonadectomy and hormone replacement

All mice between 75 and 85 days of age were gonadectomized. At the time of surgery, each mouse received a subcutaneous implant made of a 5-mm Silastic tube (Dow Corning, Corp., Midland, MI; 1.98 mm inner diameter × 3.18 mm outer diameter). Implants were either filled with 2 mg/ml 17β-estradiol benzoate in sesame oil (25 μl) or empty. During the surgery, mice were anesthetized with isoflurane. Following surgery, mice were given 0.9% sodium chloride subcutaneously and 0.25% bupivacaine as an analgesic and individually housed. All eight groups contained at least seven animals at the time of surgery.

Tissue collection

Animals were anesthetized with isoflurane and killed by cervical dislocation 3 weeks after surgery. Brains were then collected and quickly free hand dissected on ice. The cerebellum was gently separated from the inferior colliculi and brainstem, and the hypothalamus was removed from the ventral surface of the brain by gently separating it from the cerebral hemispheres, brainstem, and the optic chiasm. The hippocampus was carefully separated from the cortex, hindbrain, and diencephalon by removing the cerebral cortex with an incision at the end of the hemisphere and one 2 mm rostral to the first incision. Next, the cortex was removed, which revealed the hippocampus. All tissue was rapidly frozen following dissection to preserve for RNA extraction. Estradiol-implanted animals that were designated for Western blots had the whole brain removed and rapidly frozen on dry ice.

Quantitative real-time PCR

RNA was isolated from the brain tissue (Qiagen RNeasy Kit), and cDNA was generated from RNA by reverse transcription with Applied Biosciences High Capacity cDNA Reverse Transcription Kit. Real-time PCR was performed using the Applied Biosystems StepOne Plus for SYBR Green-based detection with Fast SYBR® Green Master Mix. Biological replicate samples of 5 ng were run in triplicate, and the average was used for data analysis. Quantification of *Gh* (F: 5′-AGGCCCAGCAGAGAACCGACA, R: 5′-ACGGTCCGAGGTGCCGAACA; source sequences: AK019954, AK030419, AL604045, BB024006, Consensus CDS: CCDS25554.1, UniProtKB/Swiss-Prot: P06880) gene expression levels was calculated based on the threshold cycle for each well using the provided software and normalized to *B2M* (F: 5′-GGCTCACACTGAATTCACCCCCAC, R: 5′-ACATGTCTCGATCCCAGTAGACGGT; source sequence: AK019389, Consensus CDS: CCDS16654.1, UniProtKB/Swiss-Prot: P01887) for hippocampus and cerebellum endogenous controls; the endogenous control for hypothalamic tissue was *Ppib* (F: 5′-TGGAGAGCACCAAGACAGACA, R: 5′-TGCCGGAGTCGACAATGAT; source sequences: AK002357, AL363449, CA321924,

Consensus CDS: CCDS23301.1, UniProtKB/Swiss-Prot: P24369, UniProtKB/TrEMBL: Q9DCY1). Melting curves revealed that only one factor per primer was amplified and that there were no measurable primer-dimers. A no-template control was also run to verify that amplification only occurred in the presence of cDNA. Both B2M (Abel and Rissman 2013, Cox, So et al. 2013, Stolzenberg, Stevens et al. 2014) and Ppib (Wolstenholme, Edwards et al. 2012, Bonthuis and Rissman 2013, Wolstenholme, Rissman et al. 2013) have been used as controls in qRTPCR in our previous studies, and both B2M (Thal, Wyschkon et al. 2008, Stephens, Stephens et al. 2011) and Ppib (Pachot, Blond et al. 2004, Cao, Luo et al. 2012) have been shown to be stable controls in other work. Furthermore, there is no statistically significant difference between groups in this study in any of the tested brain regions in terms of the amplification time of either endogenous control gene (data not shown). No sex differences have been reported for these genes, and we did not find any significant difference in the amplification time for either control gene (data not shown). The total number of individual cerebellum processed was 50 (blank: XYM $N = 7$, XXM $N = 7$, XXF $N = 6$, XYF $N = 7$; E2: XYM $N = 6$, XXM $N = 8$, XXF $N = 4$, XYF $N = 5$). The total number of individual hippocampus processed was 53 (blank: XYM $N = 6$, XXM $N = 7$, XXF $N = 6$, XYF $N = 5$, E2: XYM $N = 9$, XXM $N = 8$, XXF $N = 6$, XYF $N = 6$). The total number used for the hypothalamus was 48 (6 per group).

Western blotting

Fresh frozen brains from estradiol-treated animals were cut into 120-µM coronal sections in a cryostat and frozen on glass microscope slides, and protein was extracted from either punch of the arcuate nucleus or microdissected hypothalamus samples as described previously (Addison and Rissman 2012). For protein extraction, tissue was thawed and homogenized in 10 mM Tris, 400 mM Nacl, 1 mM DTT, 1 mM EDTA, and 10% glycerol, with 10 µl/ml protease inhibitor cocktail (Sigma) and phenylmethylsulfonyl fluoride (1 mM). After centrifugation, the total lysate protein concentrations were determined by BCA protein assay (Pierce Chemical Co., Cat#

23228.). Proteins were separated on 14% polyacrylamide-SDS gels and transferred to nitrocellulose membranes. After transfer, membranes were blocked with 5% milk and incubated with an antibody for GH (1:5,000; National Hormone & Peptide Program, CA) overnight at 4°C. After rinsing, blots were incubated for 1 h with HRP-conjugated anti-rabbit IgG secondary antibody (1:10,000; Vector Laboratories) followed by detection on X-ray film (X-OMAT) with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.). The same blots were re-probed with the monoclonal antibody against β-actin at 1:10,000 (Sigma-Aldrich Corp.). The intensities of GH and β-actin bands on individual films were measured and analyzed by densitometry with ImageJ program (NIH). Levels of GH protein were normalized to those of β-actin in each sample, and the protein amount was expressed as the ratio of GH to β-actin. For the hypothalamus, 13 individual samples only from the estradiol-treated groups were used (XYM $N = 4$, XXM $N = 3$, XXF $N = 3$, XYF $N = 3$). For the analysis of the arcuate nucleus, six animals from each genotype, all estradiol treated, were assessed.

Statistical analysis

All data were analyzed using NCSS (2001). For gene expression data, normalized gene expression was calculated using the $\Delta\Delta C$t method (Livak and Schmittgen 2001). Relative quantities (RQs) were log transformed and analyzed by ANOVA with sex chromosome complement, gonadal sex, and hormone treatment as factors. For protein data, two-way ANOVAs were used with genotype and gonadal sex as the two factors. Significant results were assessed by Fisher's exact *post hoc* tests that adjust significance levels to take multiple comparisons into account.

iii. Results

Estradiol increases Gh mRNA in the hippocampus and cerebellum

Estradiol significantly increased *Gh* gene expression in both the hippocampus and

cerebellum. FCG mice that were treated with an estradiol implant at the time of gonadectomy had higher levels of mRNA in the cerebellum than those that were given a blank implant (**Figure 22A**; $F1,49 = 8.61$, $P < 0.006$), demonstrating for the first time that estradiol increases *Gh* mRNA levels in the cerebellum. Confirming previous results in rats (Donahue, Kosik et al. 2006), estradiol-treated FCG mice had higher levels of mRNA in the hippocampus than gonadectomized mice without any hormone replacement (**Figure 22B**; $F1,52 = 5.78$, $P < 0.03$). There were no main effects of gonadal sex and sex chromosome complement, nor were there any significant interactions.

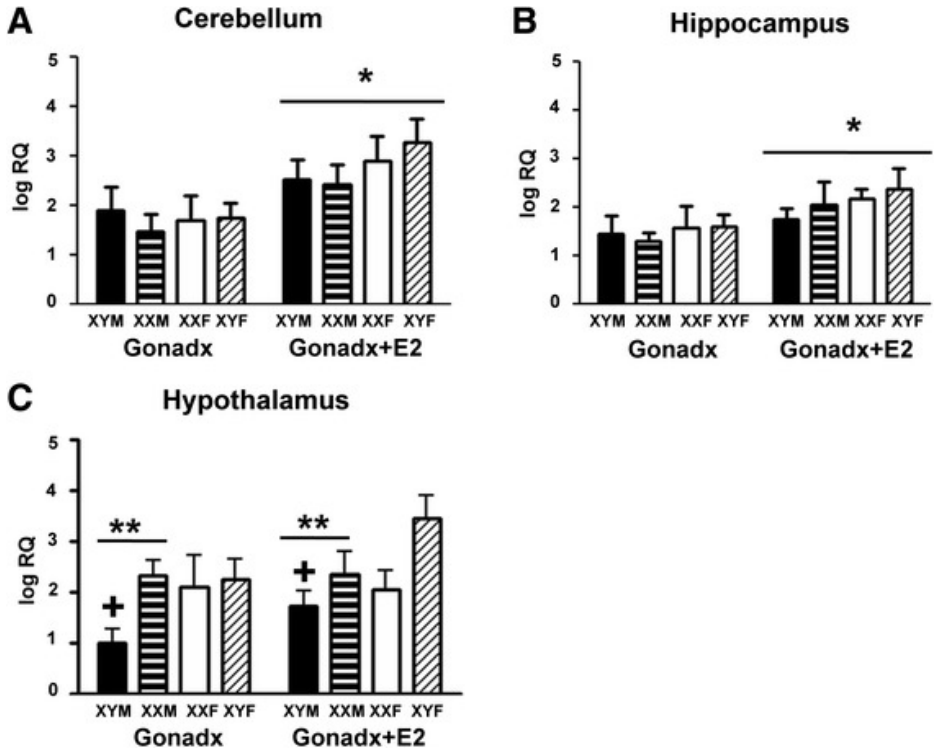

Figure 22: Gh mRNA in **(A)** the cerebellum, showing a significant increase in the relative quantity (RQ) of Gh mRNA in estradiol-treated animals of all genotypes; **(B)** the hippocampus, a significant effect of estradiol on Gh RQ was noted; and **(C)** the hypothalamus where effects of both sex chromosomes and gonadal sex were observed. Adult mice from the four core genotypes—XY males (black bars), XX males (horizontal striped bars), XX females (white bars), and XY females (diagonal striped bars)—were gonadectomized and treated with estradiol (E) or given empty implants. For the cerebellum: blank: XYM $N = 7$, XXM $N = 7$, XXF $N = 6$, XYF $N = 7$; E2: XYM $N = 6$, XXM $N = 8$, XXF $N = 4$, XYF $N = 5$. In the hippocampus: blank: XYM $N = 6$, XXM $N = 7$, XXF $N = 6$, XYF $N = 5$; E2: XYM $N = 9$, XXM $N = 8$, XXF $N = 6$, XYF $N = 6$. In the hypothalamus: $N = 6$ for all groups. The single asterisks indicate significant effect of estradiol ($P < 0.05$). The double asterisks indicate significant sex difference ($P < 0.05$). The plus symbols indicate mice in the XY male group which are significantly different from mice in the XX male and XY female groups ($P < 0.05$).

In the hypothalamus, gonadal sex and sex chromosome complement modify Gh mRNA

In the hypothalamus of FCG mice, there was a main effect of gonadal sex; males had less *Gh* expression as compared to females (**Figure 22C**; $F1,47 = 4.41, P < 0.05$). An interaction between gonadal sex and sex chromosome complement ($F1,47 = 9.21, P < 0.01$) was produced because XY female mice had higher *Gh* mRNA than XY male mice ($P < 0.05$). Animals given estradiol implants at the time of gonadectomy tended to have higher levels of mRNA than those that were given a blank implant at the time of gonadectomy, but this effect was not significant ($F1,47 = 2.69, P = 0.11$).

Sex chromosome complement modifies GH protein in the whole hypothalamus of estradiol-treated animals

In the hypothalamus, we noted an interaction between gonadal sex and sex chromosomes. Female mice had higher Gh protein than males, and XY animals had more Gh than XX mice ($F1,13 = 20.04, P < 0.002$). The amount of GH in XY female hypothalamus tissue was significantly greater than that in all other groups ($P < 0.05$). There was also a main effect of gonadal sex wherein females had more GH protein than males (**Figure 23**; $F1,13 = 9.75$, $P < 0.02$) and a main effect of sex chromosome complement ($F1,13 = 7.59, P < 0.03$) where GH levels in XY animals were greater than in XX animals.

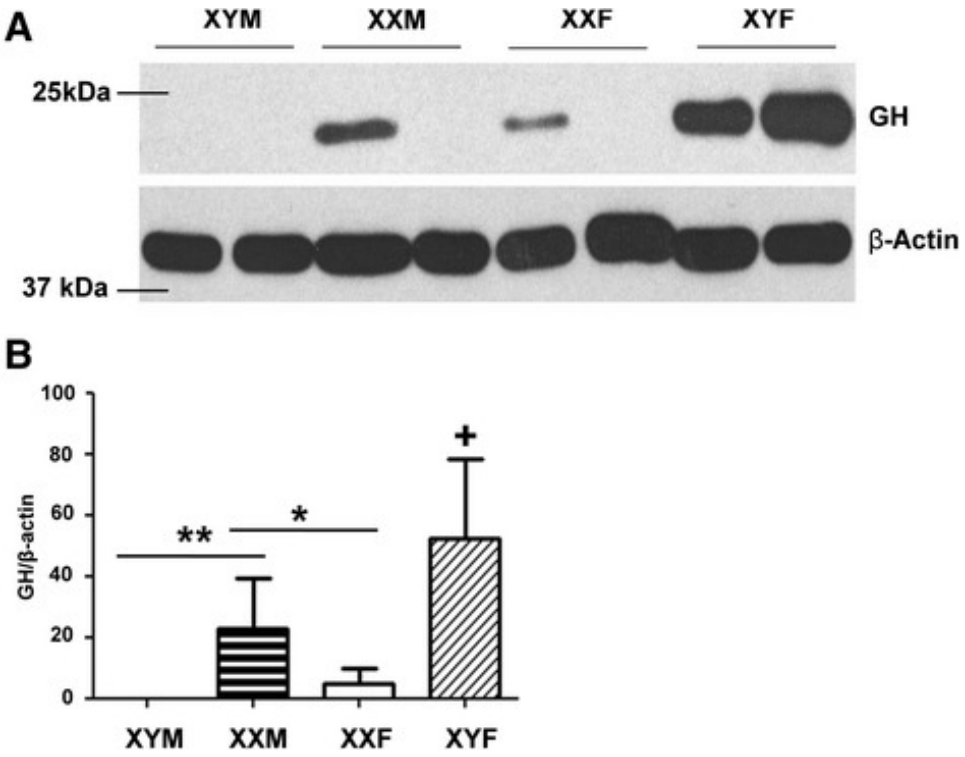

Figure 23: GH protein in the hypothalamus. **(A)** A representative blot is shown. **(B)** Densitometry data from the blots is presented. Adult mice from the four core genotypes—XY males (XYM $N = 4$, black bars), XX males (XXM $N = 3$, horizontal striped bars), XX females (XXF $N = 3$, white bars), and XY females (XYF $N = 3$, diagonal striped bars)—were gonadectomized and treated with estradiol (E). The single asterisk denotes significant effect of sex chromosome complement ($P < 0.05$). The double asterisks denote significant effect of gonadal sex ($P < 0.05$). The plus symbol denotes XY females which are significantly different from all other groups ($P < 0.05$).

Sex chromosome complement modifies GH protein in the arcuate nucleus of the hypothalamus

In the arcuate nucleus of the hypothalamus, we also noted an effect of sex chromosomes in estradiol-treated gonadectomized FCG mice (**Figure 24**; $F1,20 = 5.45$, $P < 0.04$). In this nucleus, XX mice had higher levels of GH protein than XY mice, regardless of gonadal sex ($P < 0.05$). No gonadal sex effect or interactions were present.

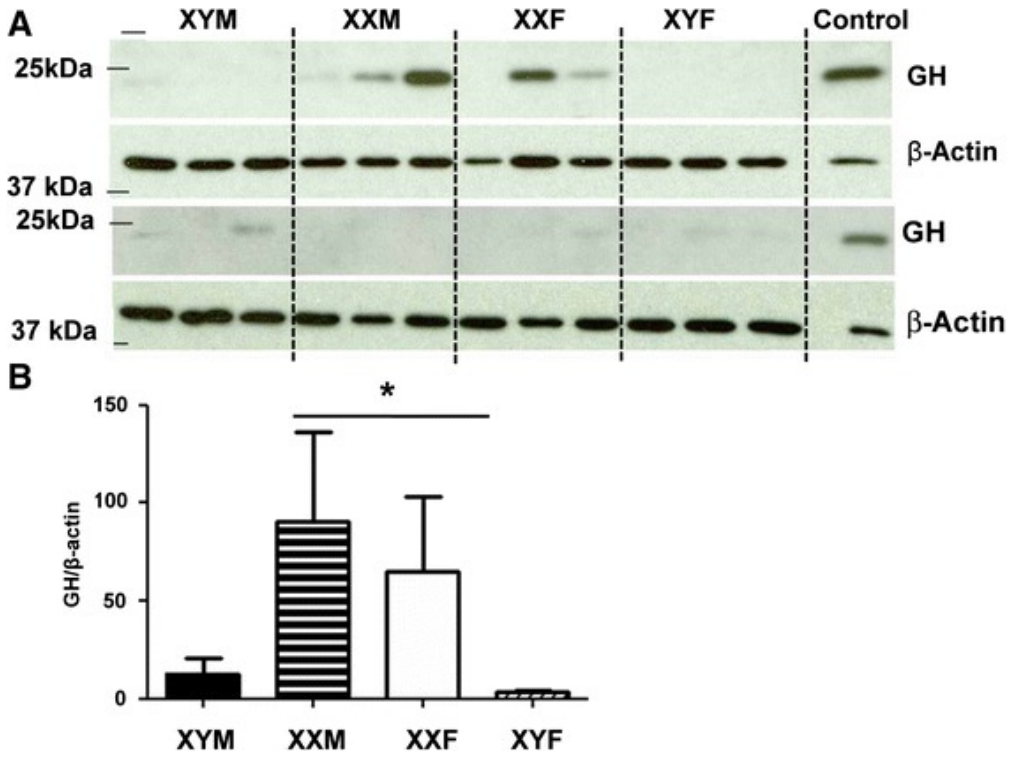

Figure 24: GH protein in the arcuate nucleus of the hypothalamus. **(A)** A representative blot is shown. **(B)** Densitometry data from the blots is presented. Adult mice from the four core genotypes—XY males (black bars), XX males (horizontal striped bars), XX females (white bars), and XY females (diagonal striped bars)—were gonadectomized and treated with estradiol (E). $N = 6$ in each group. The single asterisk signifies significant effect of sex chromosome complement ($P < 0.05$).

iv. Discussion

Our data show that estradiol treatment increases *Gh* gene expression in the cerebellum and hippocampus with a marginal effect in the hypothalamus, and that sex chromosome complement is correlated with *Gh* mRNA in the hypothalamus. Sex chromosome complement affects GH protein in the whole hypothalamus as well as the arcuate nucleus. Previous studies conducted by our lab showed that *Gh* mRNA is elevated in response to estradiol in the normal C57BL/6 female mouse hypothalamus, but not in males (Addison and Rissman 2012). In combination with the current finding that GH protein is dependent on sex chromosomes in the whole hypothalamus as well as the arcuate nucleus, we suggest that estradiol and sex chromosome genes both regulate hypothalamic *Gh* gene expression.

Evidence for direct action of estradiol on GH, and factors that mediate GH release, has existed for many years. In rats, neurons containing GH-releasing factor (GHRF) in the hypothalamus and GH cells in the pituitary co-express estrogen receptors (Shirasu, Stumpf et al. 1990). In mice, agonist binding to estrogen receptors alpha and beta induces expression of *Gh* in the pituitary (Avtanski, Novaira et al. 2014). Moreover, estradiol increases *Gh* mRNA in the mPOA and arcuate nucleus of female mice, and the estrogen receptor antagonist tamoxifen blocks this effect (Addison and Rissman 2012). Estradiol treatment also stimulates GH secretion in humans, increasing the amount of GH per pulse twofold (Painson and Tannenbaum 1991, Shah, Evans et al. 1999, Veldhuis, Anderson et al. 2004). In addition, GH receptor expression and GH binding increase in an estradiol-dependent manner in rat and human bone cell lines (Slootweg, Swolin et al. 1997), and this relationship between estradiol and GH extends to the liver (Fernandez-Perez, Santana-Farre et al. 2014) and ovaries (Hrabia, Sechman et al. 2012). It is clear from these previous findings, and more so now with the addition of this study, that estradiol has an important regulatory effect on *Gh* mRNA and GH protein. While a relationship in the hypothalamus and rat hippocampus was known, we now show that this is true in multiple brain regions, which we expect to have widespread implications.

Given the presence of sex differences in *Gh* expression across several brain areas, we speculate that GH may regulate sexually dimorphic processes throughout the body. For example, *Gh* gene expression in the brain correlates with increased food consumption and body weight in mice (Bohlooly, Olsson et al. 2005, Bonthuis and Rissman 2013) and has been implicated in feeding behavior and energy homeostasis (Sartorio, Conti et al. 1996, Bonthuis and Rissman 2013). In a previous study using FCG mice, XY mice, independent of gonadal sex, were more sensitive to the reward of palatable food intake (Seu, Groman et al. 2014), which may be representative of the differences that we saw in GH. This would likely be due to actions in the hypothalamus, particularly in the arcuate nucleus, which has a known role in metabolism and feeding behavior. In addition, GH deficiency has been linked with cognitive impairment (Sartorio,

Conti et al. 1996, Addison and Rissman 2012, Chen, McClusky et al. 2012), and previous research has shown that *Gh* expression increases in the rat hippocampus following repeated learning in response to eye-blink conditioning (Donahue, Jensen et al. 2002). In mice, infusion of GH into the hippocampus changes expression of immediate early genes and induces spontaneous locomotion, grooming, and anxiety-like behavior (Srimontri, Hirota et al. 2014). Consistent with observations in rat, our data in mice show that *Gh* is expressed in the hippocampus in a hormone-dependent manner; this may be an important aspect of learning and memory mediation by *Gh*. The cerebellum is also notably important for motor function and learning (Gruart and Yeo 1995), and we show that estradiol regulates *Gh* levels in this region too.

Pituitary GH release is sexually dimorphic (Jansson, Eden et al. 1985), and estradiol is an important activating factor in this sex difference (Birzniece, Sutanto et al. 2012). When thinking in terms of sexual dimorphism, it is imperative to consider that estradiol and other hormones are often the only contributing element. However, steroid hormones also commonly function in conjunction with, and in addition to, sex chromosome complement, which is further reinforced by the data in this study. Sex chromosome complement is an important factor for sex differences and is implicated in sexually dimorphic outcomes of aggression (Gatewood, Wills et al. 2006), social interaction (Gatewood, Wills et al. 2006, McPhie-Lalmansingh, Tejada et al. 2008, Arnold 2009, Cox and Rissman 2011), and body weight (Arnold 2009, Arnold, Chen et al. 2012, Chen, McClusky et al. 2013). Our data show the significance of sex chromosome complement for *Gh* gene expression and GH protein in the hypothalamus and the arcuate nucleus of the hypothalamus. The use of the FCG mice in these experiments demonstrates that the sex differences in these regions are not caused exclusively by estradiol, instead suggesting that these two principal mechanisms (estradiol and sex chromosomes) are acting together. We have observed a similar interaction in previous work. An interaction between ERα and sex chromosomes regulates calbindin gene expression in the prefrontal cortex and cerebellum of mice (Abel, Witt et al. 2011). Notably, *Gh* and calbindin are autosomal genes, and the mechanism driving sex chromosome

complement influence on autosomal gene expression is unclear. One strong possibility is that genes on the X chromosome that escape X inactivation modulate autosomal gene expression. Consistent with this proposition, *Gh* expression in the POA positively correlates with the combined levels of two X chromosome genes: *Kdm5c* and *Kdm6a*, which encode lysine demethylases (Bonthuis and Rissman 2013). KDM5c (lysine demethylase 5c) specifically demethylates Histone 3 Lysine 4 (H3K4) marks, acting as a transcriptional repressor. KDM6a demethylates Histone 3 Lysine 27 (H3K27) and is a transcriptional activator of gene transcription. Both *Kdm5c* and *Kdm6a* escape X inactivation dosage compensation and therefore have higher expression levels in XX females (as well as FCG XX males) (Greenfield, Carrel et al. 1998, Disteche, Filippova et al. 2002). These X chromosome genes may modulate estradiol and sex chromosome actions in the hypothalamus.

An intriguing aspect of these data is the substantial variance in the levels of *Gh* mRNA and GH protein within individuals in the genotypes that have the highest amounts of GH. This same variability was noted in our first two studies in normal C57BL/6 mice and in the aneuploid Y* mutant line (Addison and Rissman 2012, Bonthuis and Rissman 2013). Secretion of GH from the pituitary is pulsatile throughout the day (Painson and Tannenbaum 1991, Shah, Evans et al. 1999, Veldhuis, Anderson et al. 2004); therefore, we speculate that GH may be likewise produced and released via an ultradian rhythm. Moreover, this hypothesis suggests that GH produced in the hypothalamus is involved in the regulation of pituitary GH. If such a role for neural GH exists, gene and protein expression of GH in neural cells could respond to feedback from peripheral GH concentrations commensurate with secretory pulsatility. The pulsatility in GH secretion from the pituitary is sexually dimorphic, and X chromosome dosage may play a role in the dimorphism (Jansson, Eden et al. 1985) in addition to regulation by hormones. The mice in our study were gonadectomized and received an estradiol or blank implant. Thus, endogenous fluctuations of circulating estradiol do not account for the individual variation we have noted both in GH protein and mRNA.

These data indicate that mRNA levels measured in the whole hypothalamus are comparable to whole hypothalamic protein levels. However, the direction of the relationship between amounts of GH and the sex chromosomes, with XYF hypothalami containing the greatest amounts of GH and message, is in stark contrast to the data we collected in the arcuate and mPOA in normal C57BL/6 mice (Addison and Rissman 2012). In that study, females (XX) had more *Gh* mRNA than males (XY). In the aneuploidy (XY* model) mouse mutant, we measured Gh mRNA in mPOA and found a significant positive relationship between levels of *Gh* and numbers of X chromosomes (Bonthuis and Rissman 2013). Thus, our protein data from the arcuate nucleus in the FCG mice is in agreement with the data collected in normal mice. The fact that the entire hypothalamus and the arcuate are regulated differently by sex chromosome complement suggests cellular differences in the transcriptional regulation of GH. Cellular phenotypes in the hypothalamus are not uniform, and one hypothesis is that estrogen receptor alpha, which is co-expressed in nearly all GH-positive neurons in the arcuate nucleus and mPOA (Addison and Rissman 2012), interacts with sex chromosome genes that escape X inactivation.

Conclusions

We have shown that *Gh* gene expression is influenced by estradiol in the cerebellum and hippocampus. In the hypothalamus, an interaction between estradiol and sex chromosome complement was produced by the differences in XY male and female mice, with males having the lowest GH protein and mRNA and females having the greatest amounts. In estradiol-treated mice, GH protein levels in the arcuate nucleus were dependent on sex chromosome complement since XX mice of both gonadal sexes had higher levels than XY mice. This work draws attention to sex differences of *Gh* gene expression and GH protein in previously unexplored areas of the brain. Future research should focus more on region-specific roles for *Gh* in physiological processes and behavior, as well as which X chromosome genes have a role in autosomal gene expression and how they act in conjunction with steroid hormones.

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